

REVIEW

Fundulus as the Premier Teleost Model in Environmental Biology:

Opportunities for New Insights Using Genomics

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Running title: Opportunities for genomics research with *Fundulus*

Abstract

A strong foundation of basic and applied research documents that the estuarine fish *Fundulus heteroclitus* and related species are unique laboratory and field models for understanding how individuals and populations interact with their environment. In this paper we summarize an extensive body of work examining the adaptive responses of *Fundulus* species to environmental conditions, and describe how this research has contributed importantly to our understanding of physiology, gene regulation, toxicology, and ecological and evolutionary genetics of teleosts and other vertebrates. These explorations have reached a critical juncture at which advancement is hindered by the lack of genomic resources for these species. We suggest that a more complete genomics toolbox for *F. heteroclitus* and related species will permit researchers to exploit the power of this model organism to rapidly advance our understanding of fundamental biological and pathological mechanisms among vertebrates, as well as ecological strategies and evolutionary processes common to all living organisms.

Keywords: *Fundulus heteroclitus*, physiological genomics, ecological genomics, evolutionary genomics, toxicogenomics, environmental genomics

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List of Abbreviations

AHR: aryl hydrocarbon receptor; AHRR: AHR repressor; ANF: α -naphthoflavone; ARNT: AHR nuclear translocator; BAC: bacterial artificial chromosome; BAP: benzo[*a*]pyrene; bHLH-PAS: basic helix-loop-helix Per-ARNT-Sim; BNF: β naphthoflavone; CAR: constitutive androstane receptor; CFTR: cystic fibrosis transmembrane conductance regulator; COX: cyclooxygenase; CYP: cytochrome P450; DDT: dichloro-diphenyl-trichloroethane; DLC: dioxin-like compound; DO: dissolved oxygen; ECOTOX: Environmental Protection Agency's database of aquatic toxicity testing; ENU: N-ethyl-N-nitrosourea; ER: estrogen receptor; ERR: estrogen receptor-related receptor; EST: expressed sequence tag; FGC: *Fundulus* Genomics Consortium; FXR: farnesoid X-receptor; GO: Gene Ontologies; GR: glucocorticoid receptor; GST: glutathione S-transferase; HAH: halogenated aromatic hydrocarbon; HIF: hypoxia-inducible factor; KEGG: Kyoto Encyclopedia of Genes and Genomes; LDH: lactate dehydrogenase; LXR: liver X-receptor; MH: major histocompatibility ; MIS: maturation induction steroid; MR: mineralocorticoid receptor; NBH: New Bedford Harbor; NCBI: National Center for Biotechnology Information; NR: nuclear receptor; NKCC: $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter; NHE: $\text{Na}^+ - \text{H}^+$ -exchangers; P450arom: cytochrome P450 aromatase; PAH: polycyclic aromatic hydrocarbon; PCB: polychlorinated biphenyl; P_{crit} : critical oxygen pressure; PO_2 : oxygen partial pressure; PCR: polymerase chain reaction; PXR: pregnane X-receptor; SET protein: Phosphatase 2A inhibitor I2PP2A; SNP: single nucleotide polymorphism; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; VDR: vitamin D receptor

1. Introduction

Fundulus is a diverse and widespread genus of small teleost fishes, with species inhabiting coastal marshes from New Brunswick and the north shore of the St. Lawrence to Florida and the Gulf Coast from Florida to Texas, as well as inland systems of North America (Bigelow and Schroeder, 1953; Lee et al., 1980; Scott and Crossman, 1998). *F. heteroclitus*, also called the killifish, mud minnow or mummichog, is one of the most abundant intertidal marsh fishes along the east coast of North America, where they play a dominant role as both piscivore and prey for a variety of birds, fishes and invertebrates (reviewed in Able, 2002; Able et al., 2007; Kimball and Able, 2007).

F. heteroclitus is non-migratory (Skinner et al., 2005), with local sub-populations exhibiting summer home ranges on the order of 30-40 m (Lotrich, 1975) and greatly restricted winter movements (Fritz et al., 1975). This broad distribution and limited home range have made *F. heteroclitus* a powerful field model for examining biological and ecological responses to natural environmental changes, such as the wide variations in salinity, oxygen, pH, and temperature, that routinely occur in estuarine ecosystems. *F. heteroclitus* thrive in highly populated coastal areas and chemically-polluted sites where they have evolved mechanisms to tolerate some toxic chemicals. Abundance and accessibility make *F. heteroclitus* easy to collect; their small size and adaptability to a range of environmental conditions make them easy to maintain in the laboratory. All life stages are hardy and amenable to experimental manipulation. These attributes make *F. heteroclitus* a valuable laboratory model for the study of physiological processes such as osmoregulation and reproduction in aquatic vertebrates. Consequently, these organisms are used both in laboratory and field studies to examine basic disease processes and

toxicological mechanisms, as well as ecological responses associated with chemical pollutants and other anthropogenic stressors. Finally, the many related species of *Fundulus* distributed over great geographic distances provide a powerful framework for investigating fundamental ecological and evolutionary processes.

This paper reviews selected key advances in physiology, gene regulation, toxicology, and evolutionary genetics that have relied on the distinctive, often unique, attributes of *Fundulidae*. Given the scope of research that relies on *Fundulus* models, this review is not comprehensive; rather, emphasis is placed on research in which genomics-based technologies have enhanced our knowledge of fundamental biological processes. Within this perspective, we summarize genomics resources that are currently available for *F. heteroclitus*. Finally, we argue that further development of such resources will position *F. heteroclitus* and related species as a powerful model system for testing hypotheses regarding biological responses to environmental change across levels of biological organization from molecules to ecosystems.

2. Physiology

Physiological studies in *F. heteroclitus* have been aided by the species tolerance to a range of abiotic factors, ease of capture, and adaptation to laboratory conditions. As described here, research on *F. heteroclitus* has significantly increased our fundamental knowledge on the mechanisms by which fish adapt to key environmental challenges such as changes in salinity (2.1), oxygen levels (2.2) and temperature (2.3). As well, they have been used to better understand selected integrated systems, such as reproduction and development (2.4) and vision (2.5), among other physiological processes. Many of these

studies have led to important new knowledge regarding gene regulation (3.0) and responses to toxicants (4.0).

2.1. *Osmoregulation*

F. heteroclitus is renowned for its euryhaline capabilities, readily adapting to environments ranging from ion-poor to hypersaline conditions as high as 120 ‰ (Griffith, 1974). Based upon this attribute, *F. heteroclitus* has been and continues to be an important model organism for understanding mechanisms of teleost osmoregulation, as documented in two major reviews: Karnaky (1986) focused on chloride cell structure and function and Wood and Marshall (1994) compared *in vitro* and *in vivo* approaches to understanding euryhalinity in this species. Current models for teleost ion transport and acid-base regulation in gills (Evans et al., 2004; Evans et al., 2005) and other major osmoregulatory organs (Marshall and Grosell, 2005) also rely extensively on research conducted with *F. heteroclitus*.

2.1.1. *Seawater*

Keys and Willmer (1932) identified a morphologically-distinct cell type in eel gills that was rich in mitochondria that they reasoned was responsible for NaCl secretion by marine teleost gills. Philpott and Copeland (1963) recognized a field of these cells in the gills, skin and buccal epithelium of *F. heteroclitus* and described the curious ultrastructure, with a hugely expanded basolateral membrane surface in serpentine tubules that formed a mesh among the well-organized mitochondria. Na⁺,K⁺-ATPase, localized specifically on the basolateral membrane of these “chloride cells” (Karnaky et al., 1976), displayed higher activity in the gills of *F. heteroclitus* adapted to sea water than to fresh water, and higher in both conditions than in fish adapted to brackish water

resembling the ionic composition of the blood (Epstein et al., 1967; Towle et al., 1977). These observations of Na^+,K^+ -ATPase activity explained the transepithelial secretion of Na^+ , but not Cl^- exit from the animal into seawater (Silva et al., 1977).

An explanation for the movement of Cl^- against both electrical and concentration gradients, the “pump-leak” pathway, relied on two additional discoveries: a transporter that used the Na^+ gradient to drive Cl^- accumulation intracellularly and an anion channel in the apical membrane that allowed the Cl^- to leak out into the environment. Using the patch-clamp technique, Marshall et al. (1993) showed that the *F. heteroclitus* chloride cell has a channel that is similar to the mammalian cystic fibrosis transmembrane conductance regulator (CFTR). Singer et al. (1998) cloned this CFTR homolog in *F. heteroclitus*, showing that its expression increased after transfer to seawater, and that cAMP activated anion conductance by the channel when expressed in amphibian oocytes. Adaptation to seawater augments ion secretion in parallel with CFTR expression (Marshall et al., 1999) and there is mobilization of both the CFTR and $\text{Na}^+,\text{K}^+,2\text{Cl}^-$ cotransporter (NKCC) in chloride cells, placing more of the former in apical membrane and more of the latter in the basolateral membrane (Marshall et al., 2002).

Soon after Eveloff et al. (1978) first recognized the role of NKCC in ion balance in the shark rectal gland, Degnan et al. (1977) documented NKCC function in isolated opercular membranes of *F. heteroclitus*. Since then, others have shown NKCC dependence on K^+ (Marshall, 2002), inhibition by loop diuretics (Eriksson et al., 1985; Marshall and Grosell, 2005), and expression changes during adaptation to seawater (Scott et al., 2004a). Other recent studies in this area have explored the regulatory pathways involved in NKCC activation (Flemmer et al., 2002; Gagnon et al., 2006) and the role of

inducible cyclooxygenase COX-2 in seawater acclimation (Choe et al., 2006; Delpire and Gagnon, 2006). COX-2 regulates prostaglandin metabolism and mediates eicosanoid synthesis which, in turn, regulate NaCl secretion by the killifish opercular epithelium (Van Praag et al., 1987; Evans et al., 2004).

The Na⁺,K⁺-ATPase, which is responsible for producing the Na⁺ transmembrane gradient that drives NKCC operation, has been cloned from *F. heteroclitus* (Semple et al., 2002). Current evidence indicates a complicated layering of regulation at the protein and expression levels associated with adaptation to seawater (Mancera and McCormick, 2000; Lima and Kültz, 2004; Scott et al., 2004a; Scott et al., 2004b; Choe et al., 2006). The Na⁺-H⁺-exchangers (NHE), which are usually associated with acid secretion in freshwater ion and acid-base balance (Evans et al., 2005), also appear to play a role in the regulation of acid transport in marine conditions (Edwards et al., 2005).

2.1.2. Freshwater

Most of the osmoregulatory research with *F. heteroclitus* has been directed toward mechanisms accommodating increased salinities, yet the converse, adjustment to lower salinities, requires even more challenging and diverse strategies (Evans et al., 2005; Marshall and Grosell, 2005). Because freshwater habitats are geologically transient, with inland lakes and streams forming with geological changes, there have been innumerable opportunities for different freshwater osmoregulatory mechanisms to evolve. As a result, the paradigm followed by the major model species, *e.g.* rainbow trout, in which acid-secreting and base-secreting cells operate in parallel to link acid-base regulation with NaCl uptake (Evans et al., 2005), is not present in other groups. *F. heteroclitus* (Katoh et al., 2003) expresses the pivotal enzyme V-type H⁺-ATPase in the basolateral rather than

the apical membrane, as in trout (Lin et al., 1994). The seawater opercular membrane secretes NaCl, as does the gill. However, in freshwater the gills actively absorb Na^+ but not Cl^- (Wood and Marshall, 1994), while the opercular epithelium actively absorbs Ca^{2+} (Patrick et al., 1997; Patrick and Wood, 1999; Marshall and Grosell, 2005) and Cl^- (Marshall et al., 1997), but not Na^+ . Adaptation to freshwater relies on complex dynamics of Na^+ - H^+ -exchanger isoforms, Na^+ , K^+ -ATPase and carbonic anhydrase (Edwards et al., 2002; Edwards et al., 2005; Scott et al., 2005a) and likely reflects both a rapid turnover of transporting cells (Daborn et al., 2001; Laurent et al., 2006) and a Na^+ uptake system that could rely instead on Na^+ - H^+ exchange or neutral NaCl cotransport. As a result, Na^+ uptake is much less efficient than in the trout, yet it is a mechanism that still operates sufficiently to allow *F. heteroclitus* to occupy even ion-poor freshwater habitats, so long as the animals are actively feeding. Indeed the ionic contribution of food is essential for *F. heteroclitus* survival in ion-poor freshwater because Cl^- uptake is limited in the opercular membrane and absent in the gill (Marshall et al., 1997; Laurent et al., 2006).

Although the important discovery of CFTR occurred in *F. heteroclitus* before other teleosts, and other transport protein genes are now known (Na^+ , K^+ -ATPase, NHE, carbonic anhydrase, H^+ -ATPase, NKCC), many other transporter homologs and their regulatory proteins remain enigmatic. Importantly, the regulatory proteins that may be intimately associated with Na^+ , K^+ -ATPase, H^+ -ATPase, NKCC, and CFTR are not yet described. To date only three potential regulatory proteins have been sequenced, the glucocorticoid receptor (Scott et al., 2005a), COX-2 and the 14-3-3 protein (Kültz et al., 2001; Scott et al., 2004a; Scott et al., 2005a; Choe et al., 2006).

2.1.3. *Kidney and intestine*

In the marine environment *F. heteroclitus* (Malvin et al., 1980; Burgess et al., 1998) drinks to offset osmotic water loss to the environment in a reflex stimulated by angiotensins. The intestine absorbs water and ions from the seawater ingested by the animal and in the posterior portion there is also some ion secretion, specifically of bicarbonate (Marshall and Grosell, 2005). The digestive tract of *F. heteroclitus* is unusual as it lacks a stomach and is relatively short. The intestine is normally absorptive, involving NaCl absorption in parallel to water channels that impart hydraulic conductivity and allow fluid reabsorption. The kidney of teleosts is an important means for excretion of water by freshwater-adapted teleosts and in marine teleosts for the secretion of divalent ions, especially Mg^{2+} , Ca^{2+} and SO_4^{2-} (Marshall and Grosell, 2005). The *F. heteroclitus* kidney is small and has a reduced urinary bladder where ureteral urine might accumulate and be altered before release. In seawater, tubular secretion of Na^+ and Cl^- occurs as secondary active Cl^- transport with electrically coupled paracellular Na^+ transport. Electrochemical gradients for K^+ and Na^+ allow Cl^- entry across the basolateral membrane presumably *via* NKCC and results in cytosolic Cl^- concentrations above electrochemical equilibrium. Apical Cl^- secretion is *via* a cAMP stimulated conductive pathway, by an as yet unidentified anion channel (Cliff and Beyenbach, 1992b; Marshall and Grosell, 2005). In both seawater and freshwater killifish, NKCC immunoreactivity was detected in the apical membrane of the distal and collecting tubules, and in the basolateral membrane of the second segment of the proximal tubules, the major part of killifish renal tubules (Katoh et al., 2007). These results support the secretory function of the early and absorptive function of the more distal portions of renal

tubules of these euryhaline fishes.

In freshwater, the killifish proximal tubule is also secretory (Cliff and Beyenbach, 1992b), being balanced by reabsorptive NaCl transport in the distal portions of the tubule for the net excretion of dilute urine. As mentioned above, NKCC in freshwater killifish kidney have the same immunolocalization as the kidney of seawater individuals, and agreeing with the functional findings by Cliff and Beyenbach (1992a). Development of miniaturized techniques and molecular approaches that use small tissue samples will enable future studies of transporter and channel expression in the kidney, a wide-open and particularly fascinating area for study.

2.2. Responses to hypoxia

The responses of *F. heteroclitus* to changes in oxygen have received considerable attention because this species and related *Fundulidae* thrive in estuarine habitats with variable and frequently low oxygen concentrations (Diaz and Rosenberg, 1995; Diaz, 2001; Smith and Able, 2003) and because hypoxia has been shown to cause reproductive and developmental effects in several species of fishes (Wu et al., 2003; Shang and Wu, 2004; Shang et al., 2006). In comparison to other abundant resident and transient marsh fishes, *F. heteroclitus* was found to be most tolerant of low oxygen (Davanzo and Kremer, 1994; Wannamaker and Rice, 2000; Smith and Able, 2003), with little or no mortality occurring until dissolved oxygen drops below 1 mg L⁻¹ [DO of 1 mg L⁻¹ is equivalent to an oxygen partial pressure (PO₂) of 2.9 kPa at 25°C and 25-30 ppt salinity] (Voyer and Hennekey, 1972; Smith and Able, 2003). Consistent with this high tolerance of hypoxia, *F. heteroclitus* does not avoid water of 1 mg L⁻¹ in laboratory choice experiments, whereas other marsh fish show strong avoidance behaviors (Wannamaker

and Rice, 2000). *Fundulus grandis* occurs in similar habitats along the Gulf of Mexico (Timmerman and Chapman, 2004), and has a correspondingly high hypoxia tolerance (Rees et al., 2001; Love and Rees, 2002). Thus, studies of the physiological and molecular mechanisms that underlie this tolerance to hypoxia in *Fundulus* species offer unique insights into individual and population level adaptations to low oxygen.

Like most fish, *F. heteroclitus* and *F. grandis* are oxygen regulators: aerobic metabolism is independent of water PO_2 across a broad range, but becomes limited at low PO_2 . The point at which respiration becomes dependent upon ambient oxygen, the critical oxygen pressure (P_{crit}), is about 4.6 kPa ($\text{DO} \cong 1.6 \text{ mg O}_2 \text{ L}^{-1}$) in both species (Cochran and Burnett, 1996; Virani and Rees, 2000). During chronic exposure to oxygen tensions near their P_{crit} , the oxygen-binding capacity of blood in *F. heteroclitus* increases due to increases in hematocrit and hemoglobin oxygen affinity (Greaney and Powers, 1977; Greaney and Powers, 1978; Greaney et al., 1980). The increase in hematocrit is likely due to several factors, including splenic contraction and, potentially, synthesis and maturation of red blood cells stimulated by erythropoietin (Lai et al., 2006). The increase in hemoglobin oxygen affinity is mediated, in part, by a decrease in red blood cell ATP, a negative allosteric regulator of oxygen affinity in fish (Greaney and Powers, 1977; Greaney and Powers, 1978). As oxygen drops below P_{crit} , *F. heteroclitus* and *F. grandis* recruit anaerobic metabolism to augment energy production. Glycolysis is the primary pathway of anaerobic energy metabolism in fishes, and greater rates are reflected by the accumulation of lactate in blood and tissues. Increased blood and tissue lactate have been documented in *Fundulus* species after both acute and chronic hypoxic exposure (Greaney and Powers, 1978; Greaney et al., 1980; Cochran and Burnett, 1996), with as much as a

20-fold increase in blood lactate in *F. grandis* exposed to acute hypoxia (1 h, $\text{PO}_2 = 1.9$ kPa) (Virani and Rees, 2000). However, even these elevated rates of lactate accumulation only accounted for a fraction (25-50%) of decreased ATP yield from reduced aerobic metabolism. This suggests that overall metabolism (ATP turnover) is suppressed in these fish during hypoxic exposure.

Low oxygen negatively impacts growth and reproduction of *Fundulus* spp. (see also *Reproduction and Development*, above). Stierhoff (2003) observed lower growth rates in *F. heteroclitus* over a 9 day exposure to 1.0 mg L^{-1} compared to normoxic controls, most likely due to decreased food intake during hypoxia. Three other observations of this study are especially noteworthy: first, moderate hypoxia (3.0 mg L^{-1}) did not reduce growth; second, the negative effects of severe hypoxia were partly, but not completely, offset when fish were allowed access to the surface and engaged in aquatic surface respiration; and third, growth was not negatively affected when oxygen concentrations were varied between 1.0 and 11.0 mg L^{-1} on a diel cycle. This latter treatment, which simulates naturally occurring oxygen cycles, suggests that brief daily exposures to low oxygen are not detrimental to growth as long as fish experience normal oxygen levels for part of the day. In *F. grandis* exposed to chronic hypoxia (28 days at 1.3 mg L^{-1}), indices of growth and reproduction (gonad-somatic index, sex hormones, and number of eggs spawned) were lower in fish held under hypoxia compared to normoxic controls (Landry et al., 2007) suggesting a possible link to population effects of chronic hypoxia.

The effects of hypoxia on the expression of specific genes, or, more commonly, the activities of their protein products, have been documented in *F. heteroclitus* and *F. grandis*, especially with regard to their capacity for carbohydrate metabolism. Under

appropriate conditions, maximal enzyme activity reflects protein amount, and hence serves as a proxy for changes in gene expression (Pierce and Crawford, 1994). Greaney et al. (1980) found significantly higher activities of three enzymes of carbohydrate metabolism in liver, but not in skeletal muscle, of *F. heteroclitus* during chronic hypoxia. Increased activities of many but not all liver glycolytic enzymes have been documented in *F. heteroclitus* and *F. grandis* following exposure to hypoxia (Kraemer and Schulte, 2004; Martinez et al., 2006). In *F. grandis*, fewer and smaller changes in the glycolytic enzyme activities were noted in heart and brain while many enzymes were strongly suppressed in skeletal muscle (Martinez et al., 2006). In aggregate, these results demonstrate that chronic hypoxia alters the capacity for carbohydrate metabolism in *F. heteroclitus* and *F. grandis*, with the important caveat that the responses are both tissue- and enzyme-specific.

Microarrays have been used to study the effects of hypoxia on global gene expression in two other teleost fish (Gracey et al., 2001; Ton et al., 2003; Van der Meer et al., 2005). In all three studies, hypoxia resulted in changes in the levels of numerous transcripts encoding proteins involved carbohydrate metabolism, protein synthesis, cell proliferation, and motility. In contrast, a proteomic study of zebrafish skeletal muscle revealed limited changes in protein expression after hypoxia (Bosworth et al., 2005). The difference between results of microarray and proteomic studies may be due to experimental design (levels and lengths of hypoxia) or biological differences (species, tissues, or developmental stages), or could be the result of uncoupling of mRNA and protein expression under hypoxia.

2.3. Temperature adaptations

Fundulus heteroclitus is distributed along one of the largest and steepest thermal cline in the world, the eastern seacoast of North America where there is a 1°C change per degree latitude (Powers et al., 1993; Crawford et al., 1999a). Thus, fish in Maine are more than 12°C cooler than fish from Georgia. This steep thermal gradient is thought to be responsible for many of the biochemical and physiological differences among populations. For example, there are many enzyme loci where one allelic isozyme replaces another along the coast (Place and Powers, 1978; Powers and Place, 1978; Palumbi et al., 1980; Cashion et al., 1981; Van Beneden and Powers, 1985; Brown et al., 1988; Ropson and Powers, 1989; Ropson et al., 1990). For one of these loci, the heart type lactate dehydrogenase (Ldh-B), the clinal variation is selectively important (Powers et al., 1991; Crawford et al., 1999a).

2.3.1 *Ldh-B*

In the teleost fish *Fundulus heteroclitus*, the heart-type lactate dehydrogenase (Ldh-B) encodes two predominant allelic isozymes: LDH-Bb₄ (a homotetramer of one allelic form) in the northern population and LDH-Ba₄ (a homotetramer of an alternative allelic form) in the southern population (Place and Powers, 1978; Powers and Place, 1978). Sequence comparisons (Lauerman, 1990; Bernardi et al., 1993) reveal two non-synonymous changes which distinguish the two codominant Ldh-B alleles. One of these polymorphisms is located in exon 7, encoding amino acid position 311 (northern serine, southern alanine). The other polymorphism is located in exon 4, encoding amino acid position 185 (northern alanine and southern asparagine). This 4th exon amino acid substitution was found to be responsible for the difference in the thermal stability of the two LDH-B allelic enzymes (Lauerman, 1990).

The considerable evolutionary investigations of Ldh-B polymorphisms (see review by Powers et al., 1993) have focused on the apparent adaptive differences between populations in the northern and southern extremes of *F. heteroclitus*' distribution along the North American Atlantic Coast. The variations at the Ldh-B locus reflect this difference in environmental temperature: northern populations produce an enzyme with a lower K_m and greater k_{cat} when measured at low or intermediate temperatures (Place and Powers, 1979; Place and Powers, 1984), and have significantly greater levels of Ldh-B transcription that results in greater concentration of this protein (Crawford and Powers, 1989; Crawford and Powers, 1992). These differences compensate for the effect of lower habitat temperature on metabolism (Crawford and Powers, 1989) and appear to be an adaptive response to lower temperature (Place and Powers, 1979; Pierce and Crawford, 1997b; Crawford et al., 1999b). The biochemical differences in the Ldh-B enzyme are important for organismal performance. Individuals from a single well-mixed population that are homozygotic for Ldh-Bb₄ (northern alleles) swim faster at the lower temperatures than individuals homozygotic for Ldh-Ba₄, reflecting the difference in enzyme kinetics (DiMichele and Powers, 1982b). Developmental rates and metabolisms are affected by the Ldh-B genotype (DiMichele and Powers, 1982a; DiMichele and Powers, 1984a; Paynter et al., 1991) and juvenile survival is dependent on the Ldh-B genotype (DiMichele and Powers, 1991). Importantly, metabolic rates are directly affected by the Ldh-B genotype. Replacing one allelic form of the protein with another alters the rate of carbon flux (DiMichele et al., 1991).

2.3.2 Glycolysis

The Ldh-B locus is an example of biochemical adaptation. To better quantify the

frequency of adaptive changes, the variation in enzyme concentration was examined for all the eleven glycolytic enzymes within and among species of *Fundulus* acclimated to one temperature, including 14 taxa (8 species, with 2 populations from 6 species) of *Fundulus* (Pierce and Crawford, 1997b; Crawford et al., 1999a). Among these 14 taxa were 3 pairs of sister species where one species was distributed along the steep thermal cline of North American and the sister species lacked this distribution (Pierce and Crawford, 1997a). Measuring all eleven glycolytic enzymes among all these taxa allow us to test the effect of phylogeny and whether the changes in enzyme expression were adaptative or more parsimoniously described as neutral evolved changes.

Much of the variation in 7 of the 11 glycolytic enzymes is explained by phylogenetic distance. With greater evolutionary distances, there are greater differences between taxa. Thus these differences are most parsimoniously explained by random changes among lineages (Pierce and Crawford, 1997a). The amount of variation that can be explained by phylogenetic distance exceeds 70% for some enzymes. However, among the glycolytic enzymes within and among *Fundulus* species, there are significant variations in three enzymes (glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase and LDH) that cannot be explained by phylogenetic distance. These three enzymes have a pattern of variation in which species distributed along the steep thermal cline of the North American Atlantic coast have greater intraspecific variation than their sister taxa that exist in the Gulf of Mexico, where no clinal variation in temperature exists. Not only is there a difference in the amount of variation but there is a specific pattern: these three enzymes have greater concentration in taxa subjected to colder environmental temperatures. This trend -- more enzyme in colder environments -- is significant among

Fundulus species after accounting for the phylogenetic relationships. It is very unlikely that random or neutral evolutionary processes could create this pattern of variation among all the species and populations tested. The most likely, and the most parsimonious, explanation for the variation in these three enzymes is that they have evolved by natural selection.

Metabolism is most likely to be affected by the changes in glycolytic enzyme expression. To test this, Podrabsky et al. (2000) examined the variation in cardiac glucose use in southern and northern population of *F. heteroclitus*. At a single common temperature heart ventricles from northern *F. heteroclitus* utilized glucose faster than hearts from southern fish. After measuring all eleven glycolytic enzymes in these individuals, the variation in enzyme concentration of three enzymes explained most the variation in glucose utilization: glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase and LDH. These are the same loci where the variation in concentration was identified as evolutionarily important by Pierce and Crawford (1997a). These data indicate that the adaptive variation in enzyme expression affected cardiac metabolism.

These data on Ldh-B and on the glycolytic enzymes provide some of the most extensive data on biochemical adaptation to temperature. This research was made possible by the uniquely broad population and phylogenetic variation within and among *Fundulus* species.

2.4. *Reproduction and development*

The reproductive strategy of killifish demonstrates several adaptations that maximize reproductive success in the intertidal zone generally and, more specifically, at local

residence sites. Adult killifish often display semilunar reproductive (including endocrine) cycles, coincident with spring tide cycles, which synchronizes reproductive maturity and spawning with availability of specific spawning substrates that are restricted to spring tides (Taylor, 1986). *F. heteroclitus* typically deposit eggs at the high water mark during high spring tides associated with new and full moons (Hsiao et al., 1996; Taylor, 1999). Therefore, mature gametes are usually produced within 5 days of a spring tide. The maturation of eggs from vitellogenic follicles is associated with the accumulation of vitellogenin, a yolk precursor protein (Selman and Wallace, 1983; Selman and Wallace, 1986). The accumulation of vitellogenin appears to be triggered by increased levels of estrogen in the bloodstream, leading to the hepatic induction of the estrogen-responsive vitellogenin gene (Cerde et al., 1996; Hsiao et al., 1996). These vitellogenic follicles progress from early-maturing follicles and then to late-maturing follicles in response to a maturation induction steroid (MIS), 17 α ,20 β -dihydroxy-4-pregnen-3-one (Greeley et al., 1986; Petrino et al., 1993; Cerde et al., 1996; Hsiao et al., 1996). While the exact trigger for ovulation in killifish is unknown, it is thought that increases in serum levels of estradiol-17 β play a role because of an increased production of estradiol-17 β by mature follicles (Bradford and Taylor, 1987; Lin et al., 1987; Petrino et al., 1989; Petrino et al., 1993; Hsiao et al., 1996).

Eggs are laid in multiple clutches in groups of 50 to several hundred depending on the spawning substrate (Taylor, 1999). Like many other Cyprinodontiform fish, *F. heteroclitus* are oviparous with external fertilization (Meyer and Lydeard, 1993). Killifish eggs are resistant to desiccation, an adaptation that permits them to be laid at the high water mark. While it has been reported that eggs can survive aurally for 14 days or

more, a recent study has shown that this dessication resistance is only effective over short time periods of two hours or less in relative humidities of 84% or less. Nonetheless, long-term egg survival may be possible depending on the humidity of the microenvironment where eggs are laid (Preston et al., 2006). In nature, eggs hatch upon immersion in water. Nonetheless, field experiments showed that eggs exposed to continuous immersion or daily immersion by flood tides failed to develop, while those placed at the high tide mark developed and hatched within 14 days as the high tide reached them (Taylor and DiMichele, 1983).

Killifish eggs are adapted to hatch only upon immersion in water and embryo development takes only 9-15 days, therefore, eggs laid on one high tide are ready to hatch with the next tide (Taylor, 1999).. Like other teleosts, egg hatching is triggered by the release of chorionase, an enzyme found in hatching gland cells. These cells are located in the buccal cavity of the opercular epithelium, and are fully developed by approximately 9 to 10 days (Armstrong and Child, 1965; Taylor, 1999). Immediately after hatching, *F. heteroclitus* are free-swimming larvae.

There is some variation in developmental and hatching rates depending on dissolved oxygen levels, temperature, photoperiod, and latitude (DiMichele and Powers, 1984b; DiMichele and Westerman, 1997; Shimizu, 2003). For example, northern populations from Massachusetts and Delaware had median hatching times between 10.5 and 12.5 days, while a southern population of killifish from Georgia exhibited hatching times of 14-15 days (DiMichele and Westerman, 1997). However, it appears that these observed differences in hatching time due to latitude counterbalance the effect of water temperature differences across the geographic distribution of the species. DiMichele and

Westerman (1997) reported that among killifish collected from Massachusetts, Delaware, and Florida at temperatures between 15 to 30°C, those from Massachusetts reached hatching stage faster than the other populations. Additionally, eggs from Massachusetts failed to survive at 30 °C, while those from Florida failed to survive at 15°C. Thus, each population is adapted to local thermal conditions such that hatching occurs within 10-15 days. Other factors such as metabolic rate (Paynter et al., 1991), enzyme activity (DiMichele and Powers, 1991), or hemoglobin function (Powers et al., 1979) may also result in hatching time variation (Taylor, 1999). However, population -specific adaptations have likely arisen due to the small home ranges of these non-migratory fish, thus allowing for the development over multiple generations of reproductive adaptations to the temperature and climate of their immediate environment (Lotrich, 1975).

Many of the same adaptations that are useful for reproductive success in intertidal environments also make *F. heteroclitus* well-suited to laboratory experimentation. The semilunar spawning cycle continues to occur without lunar or tidal stimuli (Taylor, 1986). Thus, *F. heteroclitus* spawning cycles can be maintained in captivity and are free-running in the laboratory (Taylor, 1986; Hsiao and Meier, 1992; Hsiao et al., 1994; Hsiao et al., 1996). However, laboratory cycling causes individuals to lose their synchrony, both to high tide and lunar patterns, as there is no entrainment (Taylor, 1991). Nonetheless, a free-running reproductive cycle is advantageous as it allows for daily egg collection and frequent ovarian sampling (Hsiao et al., 1996). Predictable spawning in the field and under lab conditions provides a ready source of embryos. Females produce dozens of eggs for each of many semi-lunar cycles over several months in the field or in

the laboratory with high rates of embryonic and early larval survival (Taylor et al., 1979; Hsiao et al., 1996; Black et al., 1998a; Nacci et al., 1998; Nacci et al., 2005).

Reproduction and development in *F. heteroclitus* display several other features that continue to make the species an important model for basic and applied research. Killifish produce large (approximately 2.0 mm diameter), robust eggs that readily lend themselves to embryological and developmental studies (Trinkaus, 1963; Armstrong and Child, 1965; Atz, 1986; Weis and Weis, 1989; Trinkaus, 1998; Taylor, 1999). Eggs can be stripped from females and fertilized *in vitro* (Armstrong and Child, 1965). While these features are not different from zebrafish (*Danio rerio*), embryonic development in killifish is much more protracted (9 days versus 3 days in zebrafish), which facilitates observation and experimental intervention. These attributes have also made the embryonic development of varying populations of *F. heteroclitus* important test subjects for toxicological investigations (see below, **4.0. Toxicology**). A final advantage of killifish for reproductive, developmental, and genetic studies rests on the fact that female hybrids of *F. heteroclitus* and *F. diaphanous* reproduce clonally. This feature has not been exploited to date but could be valuable in the future for studies requiring non-recombinant genetic conditions and may also increase understanding of clonal reproduction in vertebrates (Dawley, 1992).

2.5. Sensory systems

In the estuarine environment *F. heteroclitus* is both an active predator and an important prey item. It is expected that a well-developed sensory system would be an asset to these active fish. Surprisingly, research on the sensory systems of *F. heteroclitus* has been essentially limited to its visual system. That *F. heteroclitus* are visual fish is

suggested by the enhanced overall coloration of the males during spawning season. There are even suggestions that killifish possess the ability to change color in response to background color. In addition, the eye and its many complex components, such as the retina, have been the source of discovery of unique gene isoforms, including a *F. heteroclitus* retinal specific lactate dehydrogenase-C (Ldh-C) isoform (Quattro et al., 1993).

The osmoregulatory mechanisms described for the gill and opercular epithelium (see above, 2.1. *Osmoregulation*) provide the basis for characterizing the unique mechanisms required to maintain hydration levels in the eye. In particular, the lens epithelial layer is important in maintaining tightly regulated internal ion and hydration balance in the avascular lens (for review see Delamere and Tamiya, 2004). Preliminary studies suggest that killifish lenses undergo reversible changes in opacity during seawater adaptation (Singer et al., unpublished). Likewise, abrupt changes in salinity have been associated with lens opacities and cataract formation in salmonid fish species during smoltification (Bjerkas et al., 2003). Because compromised transporter function in mammalian lens has been associated with cataract formation (Moseley et al., 2002), mechanisms used by *F. heteroclitus* to maintain lens clarity during salinity change may permit greater understanding of cataract formation.

The killifish has a wide latitudinal distribution that gives rise to both thermal and lighting gradients. Temperature plays an important role in determining the retinal rod outer segment length in *F. zebrinus* (Allen, 1995). In the bluefin killifish, *Lucania goodie*, the expression patterns of retinal UV and other photo pigments were correlated with gradients in the lighting environment (Fuller et al., 2004; Fuller et al., 2005).

Natural populations of *F. heteroclitus* are distributed over a wide, well-studied thermal gradient (for example, Fanguet et al., 2006); light intensities are expected to vary even more dramatically along the same gradient. Thus *F. heteroclitus* is an important model to investigate the evolution of vision in a biophysically-variable environment.

Several other specific visual system components have been characterized in the killifish. *F. heteroclitus* possesses four cone types including UV cones (Flammarique and Harosi, 2000). The presence of UV cones, although rare among marine teleosts, is similar to teleost fishes including cyprinids and salmonids, and may be important in increasing prey contrast as well as in conspecific communication. Additional studies have described the ultrastructure of the pineal organ of the *F. heteroclitus* and compared its dual role as a photosensor and secretory organ (Omura and Ali, 1981).

Clearly *Fundulus* species provide an unparalleled model in which to examine basic physiological processes and adaptations to environmental change. Limited genomic and proteomic efforts have been begun to examine reproduction and development, and sensory systems, as well as the adaptations to changes in salinity and oxygen pressure. Greater access to genomic resources for *F. heteroclitus* would provide the opportunity to examine the potential involvement of broad suites of genes and proteins in physiological processes, both hastening and deepening our understanding of functional plasticity among vertebrates. Many of these resources can be made readily available to physiologists because of the extensive work that has been done by others in *Fundulus* genomics, as noted in the following sections.

3. Gene regulation

The extensive body of work examining the responses of *F. heteroclitus* to environmental perturbations has contributed enormously to our understanding of basic processes of cell signaling and gene expression, including the induction of biotransformation enzymes, anti-oxidant enzymes and transporters. Many of the most prominent environmental contaminants and other physiological stressors cause changes in gene expression through activation of receptors or other ligand-activated transcription factors. Several environmentally relevant transcription factors have been characterized in species of *Fundulus*, in some cases confirming findings in other fish and in other cases providing novel insight into receptor diversity. Key examples are summarized here.

3.1. Receptors and ligand-activated transcription factors

3.1.1 Nuclear Receptors

Members of the nuclear receptor superfamily have important roles in endocrinology and, as such, have emerged as targets or potential targets of endocrine-disrupting environmental chemicals. Among these, the estrogen receptors (ERs; NR3A subfamily) have received the most attention. *F. heteroclitus*, like other fishes, differs from mammals in having two ER β paralogs (ER β a and ER β b) in addition to a single ER α (Urushitani et al., 2003; Greytak and Callard, 2007). Evidence that these ERs are involved in toxic and adaptive responses to contaminant exposure is discussed below (**4.0. Toxicology**). In addition to the ERs, *F. heteroclitus* have four ER-related receptors (ERRs; NR3B) (Tarrant et al., 2006). Although not well understood even in mammals, these enigmatic members of the nuclear receptor family are potential targets for xenoestrogens (Coward et al., 2001; Tremblay et al., 2001). cDNA fragments of glucocorticoid and mineralocorticoid receptors (GR and MR; NR3C), which are involved in stress response

and osmoregulation and also are potential targets for environmental chemicals, have been identified in *F. heteroclitus*, (Coward et al., 2001; Scott et al., 2004a; Marshall et al., 2005; Scott et al., 2005b). One group of nuclear receptors that has not yet been well characterized in *F. heteroclitus* includes families NR1H and NR1I, which contain constitutive androstane receptor (CAR), pregnane X-receptor (PXR), vitamin D receptor (VDR), liver X-receptor (LXR), and farnesoid X-receptor (FXR). These receptors have important roles in the regulation of biotransformation enzymes, transferases, and transporters in response to endogenous toxicants such as bile acids and heme breakdown products (bilirubin) as well as to xenobiotics. Studies in other fish suggest that killifish possess a PXR, LXR, and FXR, but not CAR (Moore et al., 2002; Maglich et al., 2003). *F. heteroclitus* PXR and FXR orthologs have recently been cloned (Karchner & Hahn, unpublished).

3.1.2. Basic Helix-Loop-Helix Per-Arnt-Sim family of proteins

Members of the bHLH-PAS family of transcription factors are involved in developmental signaling and in sensing environmental variables such as light, oxygen, and chemicals. The most well known chemical sensor in this family is the aryl hydrocarbon receptor (AHR), which, in mammals, exists as a single gene. The AHR regulates the expression of cytochrome P450s (CYPs) and other genes in response to planar halogenated aromatic hydrocarbons (HAH) such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polynuclear aromatic hydrocarbons such as benzo[*a*]pyrene (BAP). In both mammals and fish, the AHR is required for TCDD toxicity (Fernandez-Salguero et al., 1996; Prash et al., 2003). Early work in *F. heteroclitus* provided evidence for an AHR protein (Hahn et al., 1994; Willett et al., 1995). Subsequently, *F. heteroclitus* was

the first species in which multiple AHR genes were identified (Hahn et al., 1997; Karchner et al., 1999). AHR1 and AHR2 are highly divergent AHR paralogs that are differentially expressed (Karchner et al., 1999). Enhanced AHR diversity was subsequently found to be a general characteristic of fishes (reviewed in Hahn, 2002; Hahn et al., 2006). In addition to having multiple AHR genes, *F. heteroclitus* also possesses greater allelic diversity at AHR loci as compared to mammals (Hahn et al., 2002; Yang, 2003; Cohen et al., unpublished; Hahn et al., 2004). At the population level, in a broad survey of 23 populations from South Carolina to New Hampshire, there were more than 12 single nucleotide polymorphisms in a 512 nucleotide segment of the transactivation domain found in exon 10 of AHR1 (Cohen et al., unpublished). In another study examining full-length coding sequences of AHR1, AHR2, and AHR repressor (AHRR) in seven populations of *F. heteroclitus*, all three loci were highly polymorphic (*AHR1*: 45 SNPs; *AHR2*: 30 SNPs; *AHRR*: 38 SNPs) (Hahn et al., 2005). For *AHR1* and *AHR2*, the distribution of SNPs and inferred haplotypes (specific arrangements of SNPs on a single chromosome) varied significantly among sites.

Other bHLH-PAS proteins characterized in *F. heteroclitus* include the AHR dimerization partner ARNT2 (Powell et al., 1999), an AHR repressor (AHRR) (Karchner et al., 2002), and hypoxia-inducible factor (HIF α) (Powell and Hahn, 2002). AHRR inhibits transcriptional activation by both AHR1 and AHR2, and is induced by exposure to AHR agonists (Karchner et al., 2002). Thus, it appears to be part of a negative regulatory loop that controls the AHR signaling pathway. Although not directly a target for chemicals, HIF proteins are toxicologically relevant because of potential cross-talk

with AHR signaling during fish development (Powell et al., 2002; Powell and Hahn, 2002; Meyer and Di Giulio, 2003; Kraemer and Schulte, 2004; Prasch et al., 2004).

Evidence in mammals and selected model systems suggests that the dimeric transcription factor HIF is a master regulator of gene expression during hypoxia (Wenger et al., 2005). In mammals, at least 70 genes are known to be regulated by HIF, and the list includes genes involved in oxygen delivery (erythropoietin), vasculogenesis (vascular endothelial growth factor), carbohydrate metabolism (glucose transporters and several glycolytic enzymes), and many other functions (Wenger et al., 2005; Gorr et al., 2006). The HIF dimer is composed of an α subunit, of which there are three described forms (HIF-1 α , HIF-2 α , and HIF-3 α), and a β subunit (HIF- β , also known as the aryl hydrocarbon receptor nuclear translocator, ARNT). Oxygen-sensitivity of HIF signaling is conferred by oxygen-dependent degradation of HIF- α subunits by the ubiquitin-mediated proteosomal pathway. There are several excellent recent reviews of HIF signaling in mammals (Berra et al., 2005; Hirota and Semenza, 2005; Schofield and Ratcliffe, 2005; Wenger et al., 2005).

Homologs of HIF have been identified in a handful of teleost fish (reviewed in Nikinmaa and Rees, 2005). Three forms of α subunit have been described in *F. heteroclitus*; these apparently correspond to homologs of mammalian HIF-1 α and HIF-2 α , and a third form that may be fish-specific (Powell and Hahn, 2002 and B. Rees, unpublished observations; Law et al., 2006). Only a single form of ARNT, ARNT2, has been reported for *F. heteroclitus* (Powell et al., 1999); it interacts with HIF-2 α to bind the mammalian HIF-binding consensus DNA sequence (Powell and Hahn, 2002). Although there are several putative targets of HIF regulation in fish (Nikinmaa and Rees, 2005),

direct interaction with HIF has been demonstrated for only a limited number of fish genes (Kajimura et al., 2006). One putative target gene is the liver form of lactate dehydrogenase, LDH-B (Rees et al., 2001) one of the glycolytic enzymes up-regulated by hypoxic exposure of *F. heteroclitus* and *F. grandis*. Deletion and mutation analysis of the *Ldh-b* promoter has identified a HIF-binding region (Rees et al., unpublished). Interestingly, the DNA sequence of this region is not the consensus sequence described for mammalian target genes (Wenger et al., 2005), suggesting that a broader taxonomic view of HIF function will reveal greater diversity of HIF targets.

3.2 Cytochrome P450s

CYP enzymes catalyze oxidative transformation of the structure of organic chemicals by monooxygenase reactions, involving scission of molecular oxygen and fixation of an oxygen atom into the structure (De Montellano, 1995). Currently, there are more than 6,000 named genes in the CYP gene superfamily with members in archaea, bacteria, plants and animals (<http://drnelson.utmem.edu/P450.stats.2006.htm>). Multiple CYP genes occur in animals, and in vertebrates CYP enzymes are expressed in some cell types in all organs of the body.

Substrates for CYP enzymes include an astonishing number and diversity of moderately or highly lipophilic compounds of physiological and toxicological significance. In animals, CYP enzymes involved in the synthesis and transformation of endogenous regulatory molecules (steroids, retinoids, fatty acids etc) often have narrower substrate specificity than those CYP that also have evolved functions with exogenous toxic chemicals or xenobiotics, whether of biological or anthropogenic origin. The enzymes having broad substrate specificity with toxic chemicals are principally in CYP

gene families 1-4. CYP activities with exogenously derived substrates might be considered primarily as adaptive functions, involving the protection against toxic chemicals, although metabolism can activate some compounds to derivatives that are more reactive and more toxic (Guengerich and Liebler, 1985).

The full complement of CYP genes has not been established in killifish. To date, *F. heteroclitus* CYP genes identified by cloning and sequencing occur in twelve CYP families: CYP1, CYP2, CYP3, CYP4, CYP5, CYP7, CYP8, CYP17, CYP19, CYP24, CYP46, and CYP51. By comparison with other fish species whose genomes have been sequenced, the pufferfishes *Takifugu rubripes* and *Tetraodon nigroviridis*, and the zebrafish (*D. rerio*), we can anticipate 60 to 80 or more CYP genes in *F. heteroclitus*. We can anticipate these genes will occur in the same 18 CYP gene families seen in mammals, although only 17 of the 18 mammalian CYP families have been reported in the pufferfish *T. rubripes*. CYP39 is the only family not found in pufferfish (Nelson, 2003), although it has been detected in other fish (Goldstone, J.V. and Stegeman, J.J., unpublished).

CYP gene diversity known to date in *F. heteroclitus* varies among the gene families. Among the steroidogenic enzymes, there are duplicated CYP19 (aromatase) genes in *F. heteroclitus*, with one (CYP19A) expressed predominantly in the ovary and the other (CYP19B) in the brain (Greytak et al., 2005); in mammals there is a single CYP19. CYP19B, but not CYP19A, is estrogen-inducible and is more highly expressed than CYP19A. CYP19 gene duplicates with subdivided expression domains is a general characteristic of teleosts(Engle and Callard, 2007) , and the expressed isozymes differ in

their kinetic parameters and responses to known aromatization inhibitors and phytoestrogens (Zhao et al., 2001).

Among the toxicologically important gene families, we know most about the CYP1 genes. Among these are the CYP1A enzymes that metabolize aromatic and chlorinated hydrocarbon pollutants and that are induced by these chemicals via the AH receptor. There is a growing diversity of CYP1 family genes in fishes; recent studies show that fish express four genes in three CYP1 subfamilies, CYP1A, CYP1B1 and CYP1C1 and CYP1C2 (Godard et al., 2005); three of these are expressed in killifish (Wang et al., 2006). Functions of these newer CYP1s, and indeed most CYP, are not yet known.

There are paralogous CYP3A genes in *F. heteroclitus*, CYP3A30 and CYP3A56, with one expressed more in intestine and one in liver (Hegelund and Celander, 2003). Several other fishes have been found to have two CYP3A genes, which in phylogenetic analysis appear to have resulted from separate duplications. However, it is possible that gene conversion may be masking orthologous relationships among these CYP3As and giving the appearance of independent paralog pairs (Kashiwada et al., 2005; Goldstone and Stegeman, 2006).

In other vertebrates the CYP2 and CYP4 families show the greatest degree of diversity, and the number of CYP2 subfamilies identified in *F. heteroclitus* so far (seven) suggests that this family is highly diverse in killifish as well. Notably, some CYP2 gene subfamilies were identified first in killifish, including the CYP2Ps and CYP2Ns (Oleksiak et al., 2000; Oleksiak et al., 2003). These subfamilies are closely related to the mammalian CYP2Js, and are among the fish CYP2 subfamilies showing the greatest diversity. The functional significance of this diversity is not known; however, the

enzymatic properties of the CYP2Ps and the CYP2Js are very similar with respect to arachidonic acid metabolism, suggesting that fundamental aspects of the role of these CYPs are conserved. Finding shared synteny in other species (Goldstone, J.V. and Stegeman, J.J., unpublished) suggests that the CYP2Ps are orthologous to the CYP2Js and that the fundamental biological roles of these enzymes may be approached by studies in *F. heteroclitus*. Some CYP2s may be inducible by environmental chemicals (Peterson and Bain, 2004) and, as with other CYP, there are numerous variables also to be considered in CYP2 regulation, exemplified by studies of CYP2K activities (Haasch, 2002).

While multiple CYP genes have been identified in *F. heteroclitus* based on sequence identities, details of function and regulation are known for few of these. Functional studies have been done with microsomal preparations, and on some that have been expressed in heterologous systems, e.g., the CYP2Ns, CYP2Ps, and CYP51 (Oleksiak et al., 2000; Oleksiak et al., 2003). For *Fundulus*, fundamental questions remain concerning the identity and number of CYP, their catalytic functions, their regulation and expression, their evolution and inter-relatedness, and finally, their endogenous functions. There is likely complex sharing of functions of many CYPs with endogenous and exogenous substrates, and in multiple modes of regulation for individual genes. Thus, the same CYP that oxidize xenobiotics also metabolize a broad range of endobiotic substrates including hormones, bile acids, lipids, arachidonic acid and others, and it has been suggested that all CYP may have some endogenous function (Nebert and Russell, 2002).

The role of these receptors, transcription factors, biotransformation enzymes and their regulatory networks is still poorly understood in fish and thus remains a rich source of

research questions. The availability of improved genomic resources will provide powerful tools to understand, for example, whether receptor paralogs such as AHR1 and AHR2 (or ER β a and ER β b) regulate distinct or overlapping target genes, or whether there are ligand-specific gene expression profiles. Genomic tools also will facilitate mechanistic studies of cross-talk among receptor types (e.g. AHR-HIF, AHR-ER, ER-ERR) and how this influences adaptation to environments when multiple stressors co-occur.

4. Toxicology

Because of its ecological importance, *Fundulus* was recognized early as an important laboratory model to evaluate the toxicity of environmental pollutants (reviewed in Eisler, 1986). *F. heteroclitus* is widely used for marine toxicology studies. A search of the US Environmental Protection Agency's database of aquatic toxicity testing (ECOTOX) reveals over 1000 studies using this species. Consistent with its reputation as hardy, adult *Fundulus* are relatively insensitive to lethal effects of some chemicals. In fact, an analysis of ECOTOX reveals that *F. heteroclitus* was ranked among the most sensitive species (top 25%) for only two out of 17 chemicals tested, and typically ranked in the third quartile (50 – 75%) for sensitivity among tested species. While this sample is small and potentially biased by the use of various chemicals, exposures, and lifestages, these results suggest that chemical sensitivities for *F. heteroclitus* are not universally high but vary by compound. During early life stages, killifish are among the more sensitive fish species to the extremely toxic dioxin-like compounds (DLCs), ranking third when compared to ten freshwater fish species tested similarly (reviewed in Van Veld and Nacci, in press). However, unlike other teleost species, some populations of *F.*

heteroclitus have also demonstrated an ‘evolved tolerance’ to DLCs (see below, 5.1. *Evolved tolerance to polluted habitats*).

Because of their usefulness as a laboratory model and a representative ecological model, embryos and larvae of *F. heteroclitus* have been used to assess the toxicity of single chemicals, chemical mixtures and environmental samples (Crawford and Guarino, 1976; Weis and Weis, 1977; Sharp and Neff, 1982; Khan and Weis, 1987a; Zhou et al., 1999; Zhou et al., 2001; Couillard, 2002; Nacci et al., 2002b; Wassenberg and Di Giulio, 2004a; Wassenberg et al., 2005; Arzuaga et al., 2006; Gonzalez et al., 2006; Timme-Laragy et al., 2006). As described above (2.4. *Reproduction and Development*), embryonic development is well-characterized and easily observable. Furthermore, biomarkers of xenobiotic exposure such as CYP1A inducibility are measurable early in the embryonic period and can be quantified using traditional measures of enzyme activity (e.g., Binder and Stegeman, 1980) or immunodetectable CYP1A protein (Van Veld et al., 1992; Van Veld et al., 1997; Toomey et al., 2001). Similarly, the predominant brain form of aromatase CYP19B mRNA is a sensitive and robust biomarker of environmental estrogen in both adult and larval killifish (Greytak et al., 2005; Greytak and Callard, 2007). In addition, novel non-invasive methods have been developed for quantifying the activity of contaminant-inducible embryonic enzymes to assess variation in sensitivity to contaminants among embryos and populations (Nacci et al., 1998; Nacci et al., 2005; Arzuaga et al., 2006).

4.1. Reproductive and developmental effects

The sensitivity of *F. heteroclitus*, as well as its suitability and flexibility for field and artificial stream studies and for laboratory bioassays, has allowed demonstration of

reproductive effects at various locations, determination of mechanisms responsible for reproductive effects, and demonstration of recovery in reproductive function following process changes.

In both laboratory and field studies, killifish have been used to document effects of environmental chemicals on many different end-points of reproduction and development such as viability and fertilizability of eggs and sperm (Khan and Weis, 1987a), cytogenetic effects (Crawford and Guarino, 1976; Perry et al., 1988; Black et al., 1998a; Black et al., 1998b), embryo mortality, rate of development, developmental deformities, time to hatching and hatching success (Weis and Weis, 1974; Crawford and Guarino, 1976; Weis and Weis, 1977; Black et al., 1998a; Black et al., 1998b; Ownby et al., 2002; Boudreau et al., 2004; Wassenberg and Di Giulio, 2004b; Boudreau et al., 2005; Wassenberg et al., 2005; Gonzalez et al., 2006; Timme-Laragy et al., 2006), as well as larval growth, spontaneous activity, prey capture ability, startle response, learning ability, neurotransmitter levels (Zhou et al., 1999; Zhou et al., 2001; Weis et al., 2003; Timme-Laragy et al., 2006) and sex ratios (Matta et al., 2001). End points used to assess reproductive condition of sexually mature adults include reproductive success, circulating sex steroids, gonadal size, hepatic size, yolk protein synthesis, and genetic markers such as CYP19 aromatases, ER and vitellogenin (Black et al., 1998a; Black et al., 1998b; Monteverdi and Di Giulio, 2000; Matta et al., 2001; MacLatchy et al., 2003; Boudreau et al., 2004; Boudreau et al., 2005; Greytak et al., 2005; Greytak and Callard, 2007).

Studies on pulp and paper mill effluents have identified reproductive effects in fish, linked to the presence of endocrine-active contaminants in marine and estuarine environments (Hewitt et al., 2005). Studies in the field (LeBlanc and Bain, 1997),

artificial streams (Dubé and MacLachy, 2000) and laboratory (Dubé et al., 2002; Hewitt et al., 2002; Belknap et al., 2006; Shaughnessy et al., 2007) have identified reproduction and endocrine effects in *F. heteroclitus* as sensitive endpoints that are responsive to endocrine-active contaminants in pulp mill effluents. These studies in killifish have led the way in determining the source(s) of contaminants within the mill process as well as confirming, for the purposes of environmental protection, an effective within-mill treatment process to remove the contaminants (Dubé and MacLachy, 2001; Hewitt et al., 2002).

AHRs, ERs, or other receptors could mediate at least some effects of environmental chemicals on these reproductive and developmental end-points. Dioxins, PCBs, pesticides, plasticizers, pharmaceuticals, heavy metals and other chemicals that accumulate in the environment are known to disrupt processes of reproduction and development, even at low doses and after transient exposures, by virtue of their ability to mimic or block hormone actions on cognate receptors or otherwise alter hormone-mediated signal transduction (reviewed in Phillips and Harrison, 1999; Sumpter and Johnson, 2005). Although the “endocrine disruptor hypothesis” is still debated in the context of human health (Crisp et al., 1998; Safe, 2004), studies of field-collected *F. heteroclitus* reveal clear signs of receptor-dependent chemical effects. For example, killifish inhabiting sites contaminated by PCBs or petroleum-derived PAHs exhibit elevated CYP1A levels (Stegeman, 1978; Elskus and Stegeman, 1989; Teal et al., 1992). In *F. heteroclitus* resident in New Bedford Harbor (NBH), see below Section 5.1.2. for additional details) markers of estrogen effect (P450aromB and vitellogenin) are inappropriately elevated in males, signifying an “estrogenic environment” (Greytak et al.,

2005). PCBs, the major NBH contaminant, or their *in vivo* metabolites, are known to interact with ER. Not surprisingly, the hypothalamic-pituitary-gonadal axis is functionally impaired in NBH fish during the breeding season (Greytak et al., 2005).

Changes in reproductive parameters of sexually mature killifish at sites other than NBH have also been reported (LeBlanc et al., 1997; Rice and Xiang, 2000; McArdle et al., 2004). Additionally, reproductive and biomarker changes similar to those observed in wild-caught fish can be induced in the laboratory by administering hormonal estradiol-17 β , PCB mixtures, ethinylestradiol, BAP, 4-nonylphenol, 4-(*tert*-octyl)phenol, bisphenol-A and DDT, as well as the androgen methyl testosterone and the anti-androgen cyproterone acetate (Crawford and Guarino, 1976; Black et al., 1998a; Matta et al., 2001; MacLatchy et al., 2003; Pait and Nelson, 2003; Sharpe et al., 2004; Patel et al., 2006). Furthermore, early exposure to estrogenic compounds has been shown to cause developmental deformities in the vertebrae and cranial bones in developing *F. heteroclitus*, as well as soft tissue abnormalities (Urushitani et al., 2002).

4.2. *Effects on immunocompetence*

Fundulus was one of the first fish species used as a model to study the links between pollution, immune response, and disease susceptibility (Fries, 1986). Basic parameters of immune function, from phagocytosis and respiratory burst to antibody production, have been evaluated as biomarkers of environmental stress. In general, exposure to pollutants and other stressors led to a decrease in immune function (Fries, 1986; Faisal et al., 1991; Fournier et al., 1998; Baier-Anderson et al., 2000; Rice and Xiang, 2000; Boleza et al., 2001). However, some differences in the results between *in vivo* and *in vitro* exposures suggest modulation of certain immune parameters *in vivo* (Roszell and Anderson, 1993;

Roszell and Anderson, 1994; Roszell and Anderson, 1996), or differences in the responses to the complex mixtures of contaminants present in the field (Kelly-Reay and Weeks-Perkins, 1994). Some of these seemingly contradicting results also might be explained by the influence of the endocrine system and circadian rhythm on the immune response (Nevid and Meier, 1995; Fournier et al., 1998; Rice and Xiang, 2000). These studies point to the need to consider these factors when using parameters of the killifish immune system as biomarkers of exposure to environmental stress. Furthermore, there is a need to develop tools to evaluate parameters of both the innate and the adaptive arms of the immune system, as well as tests to evaluate the functional consequences of immune change in populations of killifish that have adapted to environmental stressors.

4.3 Carcinogenesis

F. heteroclitus is currently the only known species exhibiting neoplasia in natural populations that also is amenable to laboratory studies. The killifish is, therefore, the best available model system to verify the putative cause-effect relationship between contaminant exposure and carcinogenesis in wild fishes.

Since 1990, the association between exposure to polycyclic aromatic hydrocarbons (PAHs) and development of a suite of toxicopathic liver lesions has been investigated in *F. heteroclitus* populations in the Elizabeth River (see below, Section 5.1.3). *F. heteroclitus* inhabiting the intertidal nearshore habitat in proximity to facilities of Atlantic Wood Industries exhibit a high prevalence of toxicopathic liver disease including a suite of neoplastic and pre-neoplastic hepatocellular and cholangiocellular lesions (Vogelbein et al., 1990; Vogelbein et al., 1999), a wide range of non-neoplastic hepatic lesions (Vogelbein, unpublished), exocrine pancreatic neoplasms (Fournie and Vogelbein, 1994;

Vogelbein and Fournie, 1994) and vascular neoplasms (Vogelbein et al., 1997). Hepatocellular lesions include a suite of altered hepatocellular foci (e.g., eosinophilic, basophilic, clear cell, vacuolated, and amphophilic foci), hepatocellular adenoma, hepatocellular carcinoma and hepatoblastoma (Vogelbein et al., 1990; Vogelbein et al., unpublished). Cholangiocellular lesions include cholangiofibrosis, cholangioma and cholangiocarcinomas. Among pancreatic neoplasms acinar cell adenomas and carcinomas have been identified, and vascular neoplasms include hemangioendothelioma, hemangiosarcoma and hemangiopericytic sarcoma (Vogelbein and Zwerner, 2002). Field surveys within the Elizabeth River and in other chemically impacted parts of the Chesapeake Bay, US, indicate a strong association between PAH exposure and toxicopathic liver disease, with liver lesion prevalence and severity tracking a sediment PAH gradient.

Elizabeth River *F. heteroclitus* have developed resistance to the acutely toxic components of creosote that has, in part, a genetic basis (Mulvey et al., 2002; Ownby et al., 2002; Mulvey et al., 2003). Because offspring of these fish are highly resistant to the acutely toxic components of creosote, this has provided an opportunity to conduct long-term studies to evaluate the carcinogenicity of the river sediments near one of the wood treatment facilities. Following a year-long sediment and dietary exposure regimen, fish developed a high (40%) prevalence of altered hepatocellular foci and a moderate level of hepatic neoplasms (8.7%) (Vogelbein, unpublished). This has provided some of the strongest direct experimental evidence for a cause and effect relationship between chronic PAH exposure and fish neoplasia to date.

4.4. Toxicant-altered gene expression

Changes in gene expression in *F. heteroclitus* have been used to monitor exposure to contaminants and as an index of the adaptability of natural populations to environmental stressors. The influence of physical environmental variables and chemical contaminants on CYP gene expression were documented early in this species (Stegeman, 1979; Kloepper-Sams and Stegeman, 1989; Kloepper-Sams and Stegeman, 1992). *F. heteroclitus* has been a particularly effective model for analyzing induction of CYP1A in multiple organs as a marker of activation of the AHR, environmental exposure of animals to biochemically active levels of AHR agonists and resistance adaptation (Elskus and Stegeman, 1989; Van Veld et al., 1992; Van Veld et al., 1997; Nacci et al., 1999; Bello et al., 2001; Meyer et al., 2002). One of the recently identified members of the CYP 1 family, CYP1C1, also is inducible by aromatic hydrocarbons in *F. heteroclitus* (Wang et al., 2006), indicating possible involvement in toxic effects.

Differential display and subtractive hybridization have been used to examine up- or down-regulation of known and novel mRNAs in toxicant-exposed *F. heteroclitus*. A number of genes, including UDP-glucose pyrophosphorylase, glucose 6-phosphatase, plasma coagulation factor XI, SET protein, translocon-associated protein, and three ESTs, were differentially-expressed in *F. heteroclitus* obtained from the PAH-impacted Atlantic Wood Superfund site in the Elizabeth River, Virginia, compared to fish from a reference site (Meyer et al., 2005; see Section 5.1.3. for additional details). Alterations in novel mRNAs associated with laboratory exposure to the PAH anthracene (Peterson and Bain, 2004) and to trivalent chromium (Maples and Bain, 2004) were confirmed in *F. heteroclitus* from field sites contaminated with PAHs and chromium. Roling et al. (2004) used subtractive hybridization to correlate differential gene expression with laboratory

exposure to pyrene in *F. heteroclitus* from a PAH-impacted site. Similarly, Gonzalez et al. (2006) used subtractive hybridization to show that *F. heteroclitus* exposed to arsenic during gametogenesis had offspring that upregulated myosin light chain 2, type II keratin, and tropomyosin, which correlated to an increase in trunk curvature.

A 524 spot cDNA array was used to assess differences in fish exposed in the laboratory to chromium complexes, including Cr(III) and Cr(VI), and arsenic, and in fish collected from field sites that are impacted with metals. Differential gene expression was observed between *F. heteroclitus* exposed to Cr(III) or Cr(VI) at the no-observable and lowest-observable effect concentrations (Roling et al., 2006; Roling and Baldwin, 2006). Cr(III) altered the expression of several glutathione S-transferases (GSTs) (Roling and Baldwin, 2006), while Cr(VI) altered the expression of genes involved in growth, energy metabolism, and to a lesser extent detoxification genes. Both larval and adult *F. heteroclitus* had perturbed expression of metabolic genes, while only larvae showed changes in GST expression following Cr(VI)-exposure (Roling et al., 2006). In addition, cDNA microarrays were used in combination with chemistry data as a weight-of-evidence approach to demonstrate that remediation progressed as anticipated at a chromium-contaminated Superfund site in Charleston, SC (Roling et al., 2007).

Very recently, Fisher and Oleksiak (2007) used a cDNA microarray containing 384 metabolic genes from liver and heart to compare gene expression in brains of *F. heteroclitus* collected from three polluted sites and six reference sites. The fish had been held in the laboratory under identical conditions for one year before analysis. The results showed that up to 17% of metabolic genes had evolved adaptive changes in gene expression in the populations inhabiting the polluted sites.

Thus, while some inroads have been made in understanding responses to toxicant exposure in natural populations, the questions that remain to be addressed rely on a new generation of genomic technologies that are in early stages of development. When applied to a powerful model organism such as *F. heteroclitus* and related species, genomics-based tools can have a critical role in elucidating unique pathways of toxicant action and response, revealing information on alterations in reproductive, health and immune status, and in monitoring remediation in natural environments.

5. Ecological and evolutionary genetics

Studies with *F. heteroclitus* have provided unique insights in the area of ecological and evolutionary genetics. Here, variation among natural groups and populations has permitted an unparalleled examination of environmental influences on the genome along physiological timescales (acclimation responses) to evolutionary timescales (heritable adaptive responses). Compared to genomics studies in traditional model species, a far richer understanding of genome-environment interactions can be derived from studies of natural populations of outbred species such as *F. heteroclitus* that are routinely exposed to stressors within an ecological context. Indeed, by nature of the dynamic and variable estuarine habitat which they occupy, *F. heteroclitus* exhibits extraordinary physiological resilience and has evolved tolerances to extremes of temperature, salinity, dissolved oxygen, and pollutants. Furthermore, *F. heteroclitus* is one species among many other closely and more distantly related *Fundulus* species that vary in physiological abilities and ecological niches occupied. This network of relationships make the Fundulidae particularly powerful for evolutionary and physiological studies (Powers et al., 1991;

Crawford et al., 1999a; Whitehead and Crawford, 2006b). Here we highlight several areas in which unique attributes of *Fundulus* species as individuals and populations have proved crucial to our understanding of ecological and evolutionary genetics.

5.1. Evolved tolerance to polluted habitats

Although adaptation in the wild is difficult to prove, the occurrence of large, persistent populations of non-dispersive *Fundulus* species in highly contaminated estuaries, provides suggestive evidence of genetic adaptation (Endler, 1986). Weis and Weis (1989) reviewed much of the early literature on toxicity resistance in fish, providing some of the first and most intriguing evidence concerning the genetic, biochemical, and ecological mechanisms of tolerance in killifish. Three notable examples in which natural *Fundulus* populations exposed to chemical contaminants have provided unique insights into adaptation and tolerance are highlighted here.

5.1.1 Tolerance to mercury and TCDD (Newark, NJ, USA)

F. heteroclitus resident to Piles Creek, NJ, a small industrialized tributary contaminated with a variety of metal and organic pollutants, have been the subject of studies on the genetic, biochemical and ecological mechanisms of tolerance to the highly toxic methyl mercury. Embryos and gametes of this *F. heteroclitus* population are resistant to methyl mercury toxicity, but later life stages are not, and the adaptation appears to have a genetic basis (Weis et al., 1981; Khan and Weis, 1987b; Weis and Weis, 1989; Weis et al., 2001; Weis, 2002). The resistance exhibited by Piles Creek embryos is based in part on reduced chorionic permeability (Weis et al., 1981) and in part on an increased rate of development (Toppin et al., 1987). This altered rate of embryonic development may protect the highly sensitive embryos by decreasing the time over which

they are exposed to methyl mercury. Some evidence suggested that the resistant phenotype in the embryos appeared to be inherited primarily maternally, not paternally, and was correlated with finray count, a characteristic believed to have a large genetic component (Weis et al., 1982). However, additional evidence indicated that methyl mercury-resistant gametes were also produced by resistant adults of both sexes (Khan and Weis, 1987b).

Following reports of mercury tolerance in *F. heteroclitus* from Newark Bay, Prince and Cooper (1995a; 1995b) documented tolerance to TCDD in the same species collected from another site in Newark Bay. TCDD effects could not be accounted for by differences in TCDD uptake between fish from Newark Bay and reference sites. *F. heteroclitus* from Newark Bay were unresponsive to the induction of the AHR-mediated detoxification enzyme CYP1A following exposure to TCDD, toxicologically-similar DLCs, or other AHR agonists (Prince and Cooper, 1995a; Elskus et al., 1999; Arzuaga and Elskus, 2002), suggesting that an altered AHR pathway may be involved in resistance to DLCs. Newark Bay *F. heteroclitus* had slightly lower levels of overall AHR protein expression and DNA methylation did not appear to play a role in AHR pathway functionality in Newark Bay populations (Arzuaga and Elskus, 2002; Arzuaga et al., 2004).

5.1.2. Tolerance to PCBs (New Bedford Harbor, MA, USA)

Toxicity and tolerance to DLC has also been investigated in *F. heteroclitus* resident to New Bedford Harbor (NBH), designated a Superfund site because of widespread and very high levels of PCB contamination (Pruell et al., 1990; Lake et al., 1995; Nelson et al., 1996; Black et al., 1998a). Sediment PCB levels as high as 190,000 mg PCB/kg

sediment or 19% by weight have been documented in the most contaminated areas of NBH (Weaver, 1982). *F. heteroclitus* inhabiting sites throughout NBH are exposed to DLC (Lake et al., 1995; Black et al., 1998a) exceeding levels that were lethal to fish from clean reference sites exposed via injection (Black et al., 1998a) or diet (Gutjahr-Gobell et al., 1999). Lethal concentrations between tolerant and sensitive populations differed by orders of magnitude, and exceeded the estimated range in sensitivities for all fish species tested for this category of pollutant (reviewed in Van Veld and Nacci, in press). Despite high tissue concentrations of DLCs, NBH *F. heteroclitus* are reproductively prolific (Nacci et al., 2002c) and have high condition indices (Nacci et al., 2001). Additional evidence suggests that demographic compensation, such as increased reproductive effort and/or migration, do not play important roles in supporting a persistent population in NBH (Nacci et al., 2002b; Nacci et al., 2002c; Nacci et al., in press). The history of site-specific pollution indicates that adaptation occurred relatively recently, within the past 50 years (Nacci et al., 2002b). Two generations of laboratory-bred *F. heteroclitus* from the PCB-contaminated NBH site were similarly insensitive to the lethal effects of PCBs in relation to two generations of *F. heteroclitus* from a nearby reference site (Nacci et al., 2002a). Thus, heritable adaptation through chemical tolerance to PCBs provides the best explanation for persistence of the NBH mummichog population. In this case, the magnitude of difference in sensitivities among populations suggests that the strength of selection for this evolved tolerance can be very large, and reflects a selective force much greater than typically demonstrated by natural agents (Kingsolver et al., 2001).

DLC resistance in NBH *F. heteroclitus* involves alterations in responsiveness of the AHR pathway in adults (Bello, 1999; Nacci et al., 1999; Bello et al., 2001; Karchner et

al., 2002) as well as in embryos (Nacci et al., 1998; Roark et al., 2005). Exposure to BAP caused fewer DNA adducts in NBH fish relative to reference fish (Nacci et al., 2002c), consistent with an altered AHR pathway. Like parental fish, progeny from NBH were generally refractory to CYP1A induction suggesting that some mechanisms of tolerance are inherited (Bello, 1999). Other evidence suggested that short-term or noninheritable mechanisms may contribute to the observed differences between the NBH and reference fish. For example, freshly caught NBH fish had lower hepatic content of AHR than reference fish, but the difference was no longer detected after a 90 day depuration period in the laboratory (Bello, 1999). The DLC-tolerant phenotype of adults is not accompanied by changes in the expression of mRNA for AHR (AHR1 and AHR2), partner (ARNT), or repressor (AHRR) pathway components (Powell et al., 2000; Karchner et al., 2002). Recent studies have demonstrated that the AHR1 locus is highly polymorphic in *Fundulus*, and that there are different allele frequencies in DLC-sensitive and tolerant populations (Hahn et al., 2002; Yang, 2003; Cohen et al., unpublished; Hahn et al., 2004) but the AHR1 protein variants encoded by these alleles do not display functional differences (Hahn et al., 2004). In broad geographic comparisons of a total of 22 contaminated and reference populations, significant differences in patterns of non-synonymous versus synonymous substitutions in the transactivation region of the transcription factor AHR1 suggest that changes in selective pressures related to the AHR pathway are altering these allelic frequencies between sites (Cohen et al., unpublished). The AHR2 locus also is highly polymorphic and exhibits differences in allele frequencies among sensitive and tolerant populations of *F. heteroclitus* (Hahn et al., 2005). In light

of the demonstrated role of AHR2 in DLC embryotoxicity in other fishes (Prasch et al., 2003), AHR2 variants may be more likely to be involved in the mechanism of resistance.

In contrast to AHR expression, which does not differ in embryos from NBH compared to those from a reference site (Powell et al. 2000), ER α expression is site-related (Greytak and Callard, 2007). Paradoxically, ER α , which is estrogen-inducible in teleosts, is hyporesponsive to estradiol in NBH embryos (Greytak and Callard, 2006). It may also be relevant that estrogen induction of vitellogenin is reduced in sexually mature fish. Furthermore, *F. heteroclitus* CYP19B, an estrogen responsive gene in teleosts, was approximately two-fold higher in the brain of fish from NBH than in reference site fish (Greytak et al., 2005), while Patel et al. (2006) observed that ovarian aromatase activity was significantly decreased by 10 $\mu\text{g L}^{-1}$ BAP. Although endocrine disruptor research has focused mainly on estrogen-like chemicals and ER signaling, the possibility that killifish reproductive success is impacted by pollutants acting on androgen receptors (Sharpe et al., 2004; Boudreau et al., 2005) or thyroid receptors (Carletta et al., 2002) cannot be ruled out. By analogy to acquired resistance to AHR-mediated chemical effects in populations at this and other polluted sites, it will be of interest to determine whether long term exposure to an environment with hormone mimics leads to adaptive changes in hormonal signaling which, in turn, lead to changes in reproductive success.

5.1.3. Tolerance to PAHs (Elizabeth River, VA, USA)

F. heteroclitus has adapted to life in an inlet near Norfolk, VA, that is contaminated with very high levels of creosote-associated and other chemicals, especially PAHs (Bieri et al., 1986; Padma et al., 1998). Despite a very high prevalence of liver and pancreatic lesions and neoplasms in adults (see above, 4.3. *Carcinogenesis*), the Atlantic Wood

(AW) population appears to be thriving. Individuals are resistant to the toxicity exerted by the sediments from the site, which are teratogenic to embryos and lethal to embryos and larvae bred from adult fish collected from reference sites (Meyer and Di Giulio, 2002; Ownby et al., 2002; Meyer and Di Giulio, 2003). AW *F. heteroclitus* display deviations in several biochemical parameters that could be related to their ability to survive in their contaminated environment, including increased expression of GST and p-glycoprotein (Van Veld et al., 1991; Cooper et al., 1996; Armknecht et al., 1998; Cooper et al., 1999), increased hepatic UDP-glucuronyltransferase (Gaworecki et al., 2004), and increased hepatic antioxidant defenses (Meyer et al., 2003; Bacanskas et al., 2004). AW fish exhibit decreased inducibility of CYP1A mRNA and protein and decreased catalytic activity (Van Veld and Westbrook, 1995; Meyer and Di Giulio, 2002; Meyer et al., 2002). However, many of these changes are not heritable and thus cannot completely explain the resistant phenotype, which is at least partly heritable (Meyer and Di Giulio, 2002; Ownby et al., 2002; Meyer and Di Giulio, 2003).

While it was initially hypothesized that poor CYP1A inducibility promoted a fitness advantage via reduced metabolism of PAHs to teratogenic or lethal products, studies to assess the impacts of AW sediments on early life stage development and survival failed to support this contention (Meyer et al., 2002). Subsequent studies with pure compounds demonstrated marked synergistic toxicities between PAHs or PAH-like compounds that act as AHR agonists and other PAHs that inhibit CYP1A (Wassenberg and Di Giulio, 2004b). These studies included the model PAH-like compounds β -naphthoflavone (BNF, an AHR agonist) and α -naphthoflavone (ANF, a CYP1A inhibitor) (Wassenberg and Di Giulio, 2004a) and environmentally relevant PAHs, such as BAP (AHR agonist) and

fluoranthene (CYP1A inhibitor) (Wassenberg et al., 2005). Collectively these and other studies suggest that some PAHs exert developmental effects via activation of the AHR, and that CYP1A plays a protective role via metabolism of AHR agonists. In addition to gaining insight into the adapted AW *F. heteroclitus* phenotype, this synergy has important implications for risk assessment. PAHs invariably occur as mixtures and current approaches for estimating the ecological risks of these mixtures assume additive toxicities of mixture components (Swartz, 1999; Barron et al., 2004).

These highlighted examples document both adverse and compensatory responses to chemical exposures using wild *F. heteroclitus* populations, illustrating not only mechanisms but costs of rapid adaptation. For example, an important potential fitness cost that is hypothesized to occur in response to intense selection is reduced genetic diversity. However, evidence does not support this hypothesis for the AW (Mulvey et al., 2002; Mulvey et al., 2003) or NBH (Cohen, 2002; Mulvey et al., 2003; Roark et al., 2005; McMillan et al., 2006) *F. heteroclitus* populations. In addition, there was no loss of genetic diversity in broader geographic surveys (3-22 populations) of contaminated and reference locations in comparisons of several loci including: a portion of the AHR1 locus, the mitochondrial dloop, and MH Class II intron and exon segments (Yang, 2003; Hahn et al., 2005; Cohen et al., unpublished; Adams et al., 2006; Cohen et al., 2006; Tirindelli, 2007). While the mechanisms of adaptation in these *F. heteroclitus* populations are not known, these findings are consistent with the development of rapid resistance involving few loci, which has been noted in plants (e.g., Shaw, 1999; Nacci et al., 2002b), oligochaetes (Martinez and Levinton, 1996), and insects (for example, McKenzie and Batterham, 1994). More generally, where the genetic mechanism of adaptation is known

to be associated with few loci, the ramifications of adaptation may be predictable (Taylor and Feyereisen, 1996) and are not necessarily costly (Cousteau et al., 2000). However, a number of fitness disadvantages have been observed in *F. heteroclitus* resident in contaminated sites, including poor prey-capture capacity, reduced salinity range tolerance for egg fertilization (Bush and Weis, 1983; Weis and Khan, 1991; Smith and Weis, 1997), and increased sensitivity to low oxygen and phototoxicity (Meyer and Di Giulio, 2003). *Fundulus* laboratory and field populations provide opportunities to test whether these manifestations represent generalized or specific fitness trade-offs.

5.2. Evolution of host-parasite interactions

F. heteroclitus and related species provide an attractive model system to investigate key questions regarding the evolution and ecology of host-parasite interactions in natural populations. Long-term surveys have documented the prevalence and intensity of parasite infections in populations of *F. heteroclitus* and other estuarine *Fundulus* species (Harris and Vogelbein, 2006). Ecological studies in killifish include investigation of the influence of biotic and abiotic factors (*e.g.* salinity, streamflow) on parasite distribution and abundance (Marcogliese, 1995; Janovy et al., 1997; Janovy, 2002). Killifish have also been used as model species to investigate the impacts of parasitism on the host. The trematode worm *Crassiphiala bulboglossa*, a parasite with an indirect life cycle for which fish are intermediate hosts, has been shown to affect the behavior of both infected and non-infected banded killifish *F. diaphanous* (Krause and Godin, 1994). Experimental infection with *C. bulboglossa* negatively affected the escape response of killifish to a predator and infected *F. parvipinnis* exhibited behaviors which made them more susceptible to predation by the definitive avian host, such as surfacing, shimmying, and

jerking (Lafferty and Morris, 1996). Non-infected killifish can detect infected killifish by the presence of black spots on the skin, and fish prefer individuals that are not parasitized (Krause et al., 1996). These observations led Krause et al. (1999) to hypothesize that trematode infection could affect shoal composition in killifish. Consistent with this hypothesis, distribution of parasites in shoals is non-random, indicating that non-parasitized fish tend to prefer shoals with non-infected fish (Krause et al., 1999). Parasitism also affected shoal behavior; parasitized fish tend to occupy the front and periphery of shoals, and shoals with parasitized fish have a different geometry than shoals with non-infected fish (Ward et al., 2002). Finally, the presence of parasites in killifish has been used to indicate the presence of definitive hosts in an area (Marcogliese and Scholz, 1999), as indicators of changes in the food web, and as bioindicators of ecosystem health (Marcogliese and Cone, 1997; Marcogliese, 2005). For example, the presence and abundance of the nematode *Eustrongylides ingotus* in killifish, as well as mortalities in the definitive avian host, were higher in sites with higher organic loads, probably due to increased abundance of the first intermediate oligochaete host (Spalding et al., 1993). *F. heteroclitus* from the heavily polluted NBH showed unusual patterns and rates of parasitism, including higher prevalence and intensity of the trematodes *Ascocotyle tenuicollis* and *Stomachicola rubea* (Cohen, 2002; Hicks and Steele, 2003; Cohen et al., 2006) as well as parasites outside their normal range of occurrence (Huber and Overstreet, unpublished). Others have found that *Fundulus* species resident in another highly polluted site harbored fewer parasites than those from local reference (uncontaminated) sites (Bergey et al., 2002). These findings have been interpreted as evidence of pollution-related disturbance to the complex life cycle typical of many

parasites. These observations also demonstrate that resident *Fundulus* populations provide unique and appropriate field models to test hypotheses linking direct and indirect effects of pollution to parasitism and its outcome on population persistence.

F. heteroclitus have also been used as a model to determine how selective forces such as parasite population and disease dynamics, determine and maintain diversity of the Major Histocompatibility (MH) class II genes; genes that play an important role in immune system regulation. Cohen (2002) found high levels of diversity and population-specific differences in the patterns of DNA substitution in the peptide binding site of MH class II genes in *F. heteroclitus* from the heavily polluted NBH (see above, Section 5.1.2.) and reference sites. Consistent with the potential immunosuppressive effects of contaminants but also consistent with altered parasite abundance in this highly disturbed ecosystem, NBH *F. heteroclitus* also showed higher prevalence and intensity of parasites (Cohen, 2002; Hicks and Steele, 2003; Cohen et al., 2006). However, fish from NBH were more resistant to acute experimental infection with a bacterial pathogen, *Vibrio harveyi*, than fish from reference sites (Nacci et al., unpublished). These apparently conflicting observations point to the need for a comprehensive ecological and evolutionary approach to the study of these populations, one that considers the direct and indirect effects of pollutants on both the pathogens and the fish host.

5.3 Evolutionary variation in glycolytic enzyme expression

One of the dominant environmental parameters affecting *F. heteroclitus* is the clinal variation in temperature along the eastern seacoast of North America. *F. heteroclitus* populations near the northern extreme of its distribution experience mean annual temperatures approximately 14°C colder than southern populations. The two most

important explanations for the compensatory changes are changes in the protein sequences or in the levels of expression for enzymes that affect metabolism. The primary structure for enzymes are different among populations (Place and Powers, 1978; Powers and Place, 1978; Palumbi et al., 1980; Cashion et al., 1981; Brown et al., 1988; Van Beneden and Powers, 1989; Lauerman, 1990; Ropson et al., 1990; Pierce and Crawford, 1997a). The best example of functionally important variation in protein primary structure is the heart-type lactate dehydrogenase (LDH-B) (Powers et al., 1993).

Although differences in the protein primary structure can drive biologically important changes that affect enzyme kinetics, much more of the difference between the northern and southern LDH-B allozymes is due to differences in gene expression. For *Ldh-B*, acclimation to a 10°C temperature decrease is associated with a 1.4 fold increase in expression (Place and Powers, 1984; Segal and Crawford, 1994; Pierce and Crawford, 1997b; Crawford et al., 1999a). Fixed differences between populations account for an additional 1.8 fold difference (Crawford and Powers, 1989; Segal and Crawford, 1994). The combination of physiologically-induced changes and evolutionary adaptive changes in gene expression explains a 2.5-fold difference between enzyme activities among fish from northern versus southern populations at their respective native temperatures (Crawford and Powers, 1989; Segal and Crawford, 1994; Crawford et al., 1999a). The difference in expression accounts for 70% of the compensatory changes for differences in native thermal environment and the remaining 30% is due to differences in kinetic parameters, pH and solute concentration.

The difference in LDH-B protein concentration among populations is due to an increased rate of RNA transcription in the northern populations (Crawford and Powers,

1989; Crawford and Powers, 1992). This variation in transcription of *Ldh-B* is associated with a few nucleotide changes in the proximal promoter (Segal et al., 1996; Schulte et al., 1997; Segal et al., 1999; Schulte et al., 2000). These changes in DNA sequence affect transcriptional binding sites thereby altering binding affinities (Segal et al., 1999) and impacting gene expression *in vitro* (Segal et al., 1996; Crawford et al., 1999a; Segal et al., 1999). More importantly, the pattern of nucleotide variation is non-random. There are too many fixed differences between populations at regulatory sites. This non-random pattern suggests that the nucleotide changes that affect gene expression are evolving by natural selection and, thus, are biologically important.

In summary, these changes in gene expression appear to be biologically important because they compensate for the change in thermal environment, affect physiological function, and are evolving by natural selection (Powers et al., 1991; Pierce and Crawford, 1996; Pierce and Crawford, 1997a; Crawford et al., 1999a; Crawford et al., 1999b; Oleksiak et al., 2005)

5.4. *Evolutionary variation in messenger RNA expression*

A small but influential series of microarray studies, using a variety of analytical approaches and a variety of taxa including *Fundulus* species, have contributed important data toward understanding the relative influences of different evolutionary forces on regulatory evolution (recently reviewed in Whitehead and Crawford, 2006b). The *Fundulus* studies in particular have indicated extensive variation in gene expression both among individuals within populations and between populations, and have considered taxonomic variation in gene expression within a phylogenetic and ecological context (Oleksiak and Crawford, 2002; Oleksiak et al., 2005; Whitehead and Crawford, 2006a).

Studies of gene expression evolution using *Fundulus* offer several advantages over other models. First, within-population variation can be measured, whereas individuals of many other model organisms such as yeast, worms, and fruit flies are so small that it has to date been difficult or impossible to obtain sufficient RNA to quantify inter-individual variation. Upwards of 70-95% of genes in *Fundulus* vary significantly in mRNA concentration among individuals. Since it is generally agreed that much of the variation in gene expression is heritable (Stamatoyannopoulos, 2004; Gibson and Weir, 2005; Whitehead and Crawford, 2005; Whitehead and Crawford, 2006a), such variation is likely to provide considerable raw material for evolution. Second, the ecological basis for genomic variation is clearer for comparisons among *Fundulus* taxa than for other popular comparisons such as those between humans and chimpanzees (Enard et al., 2002) or between mice and humans (Lemos et al., 2005) or among strains of fruit flies (Rifkin et al., 2003). Finally, evolutionary studies using *Fundulus* populations offer the advantage that this is a group of relatively closely related yet adaptively divergent taxa. That is, niche breadth and physiological tolerances vary extensively among species of the same genus, and even among populations of the same species. When comparing taxa that are more distantly related, the influence of genetic drift on trait variation becomes complex and difficult to interpret.

5.5. *Adaptive patterns of DNA variation*

Adaptive patterns of DNA variation have been detected in *Fundulus*. For example, polymorphisms derived through natural selection have been identified in LDH-B protein (Crawford et al., 1989; Lauerman, 1990; Powers et al., 1993), in LDH-B proximal promoter (Segal et al., 1996; Segal et al., 1999), in transactivation region of the AHR1

transcription factor (Cohen et al, unpublished data), and in the *Mhc* class II B locus (Cohen, 2002; Mulvey et al., 2003; Roark et al., 2005; McMillan et al., 2006), and non-neutral patterns of allelic distribution have been detected for several enzymes (Place and Powers, 1978; Powers and Place, 1978; Palumbi et al., 1980; Cashon et al., 1981; Brown et al., 1988; Van Beneden and Powers, 1989; Lauerman, 1990; Ropson et al., 1990; Pierce and Crawford, 1997a). These existing studies have targeted discrete candidate loci because of the specific nature of research questions being addressed, and because of lack of availability of more extensive genomic resources. This literature provides a strong foundation on which to expand the identification of adaptive variation in *Fundulus* given future expansion of genomic resources.

The availability of full genome sequence information has facilitated efforts to identify genomic regions that are adaptively significant in humans (Payseur et al., 2002; Kayser et al., 2003; Voight et al., 2006) and in species of agronomic importance (Vigouroux et al., 2002). Expansion of tools applicable to species beyond traditional genomic models holds promise for extending these findings in humans and crop species by establishing the ecological context within which genomes are shaped. For example, genome scans have recently been applied to identify candidate loci responsible for local adaptation among populations of the common frog and among populations of deer mice distributed across altitudinal gradients (Storz and Dubach, 2004; Bonin et al., 2006), and among ecotypes of lake whitefish (Campbell and Bernatchez, 2004). In the context of *Fundulus* species these approaches hold promise for identifying genes responsible for adaptation to diverse ecological variables such as pollutants, temperature, salinity, and oxygen. Furthermore, plasticity itself is an evolvable trait (Schlichting and Pigliucci, 1998), and due to the well-

documented resilience of killifish, genomics research in this taxonomic group holds promise for yielding insights into the genomic architecture that enables physiological plasticity itself.

Genomic technologies are enabling exploration of the underpinnings of acclimation and evolutionary change, driving the emergence of a new field: environmental genomics. As applied to traditional model organisms, environmental genomics has provided some key insights as to how the genome interacts with, integrates cues from, and is changed by the environment. Genomic technologies offer the opportunity to discover not only the mechanistic basis of adaptation, but additionally may hold promise for identifying the molecular basis of fitness tradeoffs. For example, upregulation of a certain signaling pathway could be necessary for chemical tolerance, whereas downregulation of the same pathway could be necessary to optimize another fitness-related trait. Though regulatory conflicts through antagonistic pleiotropy are presumed to underlie some life-history tradeoffs, few studies have identified the molecular basis for such tradeoffs (Bochdanovits and de Jong, 2004).

Expansion of *Fundulus* genomic resources to enhance the genomic toolkit and to make resources accessible to the larger research community will accelerate progress toward understanding the genomic underpinnings of adaptation to suites of toxicological and ecological stressors. Large scale genomic resources, such as a sequenced and annotated BAC library, coupled with other genomics-based tools including fine-scale genome-wide microsatellite markers and restriction-site polymorphism markers, will make it possible to conduct high-resolution genome scans for environmentally-relevant genes, greatly aiding molecular evolutionary and population genetic approaches. Finally,

a broader suite of genomic tools for *Fundulus* will provide a powerful system to investigate genetic changes occurring in wild populations subject to intense and rapidly changing environmental pressures, including a fuller understanding of the mechanistic bases of adaptation and fitness tradeoffs including the role of gene expression conflicts in antagonistic pleiotropy.

6. *Fundulus* genomics: current status

The scientific community is now in a powerful position to identify and make sense of variation within and among populations by employing genomic technologies. Genomics is the field of research, or set of tools, that examines most, if not all, of the DNA, genes, proteins or other products derived from the heritable material of the cell. The two most frequently identified tools for genomics are the sequences of the nuclear genome, which is approximately 1.5 billion basepairs per haploid genome in *F. heteroclitus*, and expressed sequence tags (ESTs). ESTs represent short, 500-700 basepair sequences from mRNAs reverse-transcribed to complementary DNAs.

6.1. ESTs

Expressed mRNAs are much less complex than genomic DNA because they lack introns, promoters and other non-transcribed DNA and only a subset of genes are expressed in a single tissue. This simplicity allows one to more readily define the proteins expressed by a gene and provides some hints about their functions. For these reasons, isolation and sequencing of mRNAs often are used to investigate genomes.

For *F. heteroclitus* there is a large collection of ESTs and complete cDNAs (Paschall et al., 2004). The National Center for Biotechnology Information lists approximately

55,000 nucleotide sequences. This is about 1/20 the number of ESTs as zebrafish (Table 1). These *F. heteroclitus* ESTs form approximately 18,000 unique sequences (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=killifish>). Transcript-based UniGene analysis at NCBI via staged clustering yields 3,415 unigenes for this dataset (<http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=8078>). Most of the *Fundulus* sequences are from two laboratories: Crawford & Oleksiak at the University of Miami ($\approx 48,000$) and Towle at Mount Desert Island Biological Laboratory ($\approx 7,000$). These ESTs represent most tissues (brain, blood, bone, eye, fat tissue, gill, gall bladder, gut, heart, intestine, kidney, liver, opercular epithelium, skeletal muscle, skin, spleen) from several populations exposed to different salinities, temperatures, hypoxia and hydrocarbons. All developmental stages and fry were also used. This diversity of tissues from fish of many populations exposed to a variety of physiological stressors should provide a broad assortment of expressed genes. The information about these ESTs includes annotations based on similarity to human, other fish and data listed in SwissProt, gene abbreviations, enzyme commission numbers, Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways and Gene Ontologies (GO) (Paschall et al., 2004).

6.2. Microarrays

There are a number of primary research publications that have used *Fundulus* microarrays produced from ESTs. As documented elsewhere in this review, these publications have been used to measure individual variation in gene expression, to examine the affect of polymorphism on tissue specific expression on cardiac physiology, to study alterations in gene expression due to toxicant exposure and to explore evolutionary and ecological processes in *Fundulus* species. These microarray studies

require appropriate bioinformatics. There are two sources of bioinformatics for most of the 55,000 ESTs: *FunnyBase* <http://crawford.rsmas.miami.edu/> (Paschall et al., 2004) and *DFCI Killifish Gene Index* <http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=killifish>. Using good annotation to know which genes belong to which metabolic pathway allowed the determination of the relationship between cardiac metabolism and gene expression (Oleksiak et al., 2005), demonstrated that a higher frequency of oxidative-phosphorylation genes are affected by stabilizing selection (Whitehead and Crawford, 2006a), and provided insight into tissue specific gene expression (Whitehead and Crawford, 2005).

6.3. Genomes

Comparative genomics, the use of other genomes from diverse species, is one of the most effective methods to address the difficulties in understanding genomes. Genomics of fishes seek to provide evolutionary comparisons to better annotate genes (providing information on function), define genomic architecture and roles of genes, and develop insights into the creation of novel structures. Fish genomic sequences were used to identify many human genes, and these were used as one of the primary pieces of evidence that the vertebrate genome had 30,000-40,000 genes and not 70,000-140,000 (Roest Crollius et al., 2000).

Currently, DNA sequences among ray-finned fishes include approximately 5.4 million nucleotides that code for approximately 160,000 proteins, from 13,000 taxonomic groups (National Center for Biotechnology Information, NCBI). There are 349 fish genomes in the NCBI database. Most of these are mitochondrial genomes rather than nuclear ones. Of the fish gene sequences, 164,109 are unigenes. Unigenes attempt to

distill the large number of ESTs encoding the same protein to a single entry. Unigenes do reduce the number of “genes” but fail to put all appropriate sequences together. For example, there are nearly 400,000 human unigenes for the approximately 25,000 genes.

The *F. heteroclitus* genome is not currently sequenced. Although there are many nuclear markers, such as microsatellites and amplified fragment length polymorphisms (Crollius et al., 2000; Adams et al., 2006; McMillan et al., 2006) and a good deal of knowledge concerning specific loci (Crawford and Powers, 1989; Bernardi et al., 1993; Crawford et al., 1999b; Karchner et al., 1999; Schulte et al., 2000; Schulte, 2001), *F. heteroclitus* lags behind many other vertebrate systems in the production of a genome sequence. This is currently being addressed. A BAC (bacterial artificial chromosome) library has been constructed by the Amemiya laboratory at the Benaroya Research Institute (www.benaroyaresearch.org) using high molecular weight DNA isolated from the erythrocytes of an individual *F. heteroclitus* collected in Block Island, RI, a location situated within the northern population range of *F. heteroclitus*. In general, northern populations of *F. heteroclitus* show lower genetic variation than southern populations (e.g., Powers and Place, 1978; Crawford et al., 1999a; Adams et al., 2006). The genome size of *F. heteroclitus* has been estimated at 3 pg per diploid nucleus, based on bulk fluorometric analysis (Hinegardner, 1968; Hinegardner and Rosen, 1972), while Gómez-Chiarri and Amemiya (unpublished) employed flow cytometry and a chicken standard to estimate the genome size at 2.75 pg per diploid nucleus. The library consists of more than 92,000 clones with an average insert size of 140 kb, resulting in an estimated 10 fold coverage of the *F. heteroclitus* genome. The availability of this library will greatly facilitate genomic research in *Fundulus* species.

6.4. Natural and constructed genetic modifications

As emphasized above, wild populations of *Fundulus* spp. provide truly unique models of genetic and genomic variation. These populations provide natural genetic variants to explore functional differences in responses to stressors such as temperature (e.g., Crawford and Powers, 1989; Schulte et al., 1997; Whitehead and Crawford, 2006a; Schulte, 2007) and chemical pollutants (Nacci et al., 2002b). In addition and despite the lack of a full genomic sequence for *F. heteroclitus*, laboratory-constructed lines are being developed to address specific research needs. Progress is being made in the development of genetically modified strains, inbred lineages, clones, mutants, small interfering RNA- and morpholino antisense-based gene knockdown and transgenics for this species. These approaches, which will be enhanced by full genome sequencing, provide powerful methods for broadly understanding the molecular biology of this species, as well as for elucidating mechanisms underlying phenomena such as adaptations to environmental stressors and modes of toxicity.

6.4.1. Transgenics

The first transgenic *F. heteroclitus*, developed as a model for in vivo mutagenesis research using the bacteriophage *phiX174*, demonstrated the feasibility of all aspects of transgenesis including successful transfer of DNA constructs via cytoplasmic injection of 1-2 cell stage embryos, genomic integration and germline transmission of a transgene (Winn et al., 1995). Most importantly, the spontaneous mutant frequencies of the mutation target gene in the transgenic *F. heteroclitus*, a measure of the balance between DNA damage and repair manifested as the accumulation of background mutations and, the measure of relative sensitivity of the target gene, were comparable to those observed

in transgenic mice that carried the identical mutation target gene (Burkhart et al., 1993). Efforts are currently underway to develop a transgenic *F. heteroclitus* using the identical λ LIZ bacteriophage shuttle vector containing the *cII* and *lacI* mutation target genes carried by the leading rodent mutation model (Kohler et al., 1991) and the λ transgenic medaka (Winn et al., 2000). There appear to be few, if any, technological challenges unique to *F. heteroclitus*, or to fish in general, to impede generation of transgenic lineages, with the exception of a limited genomic database.

6.4.2. Clones

Although natural clones of *F. heteroclitus* can be obtained (Dawley, 1992), the ability to generate clonal individuals in the laboratory from specific populations of interest can provide added benefits. In ongoing studies, clonal *F. heteroclitus* have been produced using inactivation of spermatozoa using by UV radiation, followed by precisely timed pressure treatment to produce a diploid gynogen or clone (Winn, unpublished).

6.4.3. Morpholinos

Morpholinos designed to transiently block translation of specific mRNAs have proven very powerful for studying the function of specific genes during development in zebrafish and for elucidating mechanisms of chemical effects on development in this species. For example, zebrafish morpholino studies (Billiard et al., 2006) were employed to confirm that the AHR mediates the deleterious effects of certain PAHs on the developing cardiovascular system and that inhibition of CYP1A greatly enhances this effect. This synergy was first suggested by more traditional, and less conclusive, biochemical studies in *F. heteroclitus* (Wassenberg and Di Giulio, 2004a; Wassenberg and Di Giulio, 2004b). While morpholino technology recently was shown to work in *F.*

heteroclitus (Matson and Di Giulio, unpublished observations with CYP1A morpholino), routine employment of this powerful technique for less well-studied genes will have to await sequence data.

6.4.4. Targeted mutagenesis: knockout mutants

Reverse genetics or knockout approaches are designed to assign biological function to uncharacterized genes in a genome. This efficient target-selected gene inactivation approach, demonstrated in zebrafish (Wienholds et al., 2002; Wienholds et al., 2003) and, more recently in medaka (Taniguchi et al., 2006), provides a powerful means to explore gene function by retrieving mutants with complete loss-of-function phenotype. To generate knockouts males are exposed to the potent mutagen ENU (N-ethyl-N-nitrosourea), crossed with wild-type females, and then the induced mutations are retrieved from a large library of F1 progeny using PCR-based amplification of target genes of interest, followed by mutation discovery by dideoxy resequencing.

Homozygous mutants can be generated by the F3 generation. The development of knockout mutants has promise for providing significant expansion of tools available to a broad research community as is similarly being realized in zebrafish and medaka. Further development of a *Fundulus* genetic database will be a key impetus to these efforts.

There appear to be few impediments to further development of new tools to improve our understanding of disease processes, adaptation, mutations, cancer, resistance to toxicant exposure and others. Clearly, the expansion of the *Fundulus* genomic database would facilitate the generation of new models that can provide rapid advances in key research areas in which this species has proven to be an ideal biological model.

7. Summary

The unique attributes of *F. heteroclitus* have provided a wealth of basic and applied insights into physiological, molecular, toxicological, ecological, evolutionary and disease processes that operate to shape diversity and resilience of natural populations.

Accordingly, *F. heteroclitus* is an unparalleled laboratory and field model for studying physiological plasticity, and for dissecting the bases for sensitivity or tolerance to stress.

This review highlights how *Fundulus* genomics has begun to serve research fields such as physiology, toxicology, and evolutionary genetics. *Fundulus* research has also contributed importantly to our understanding of estuarine and coastal ecology (see Able, 2002; Able et al., 2007; Kimball and Able, 2007). We envision that genomic tools have enormous potential as complementary measures permitting *Fundulus* researchers to link responses from controlled laboratory to complex field environments across levels of biological organization from molecules to populations and communities.

On May 4 – 5, 2006, a group of 35 scientists came together at the Hollings Marine Laboratory in Charleston SC for a *Fundulus* Genomics Strategy Workshop. The goal of the workshop was to facilitate the implementation of genome-enabled technology across the many disciplines in which *F. heteroclitus* serves as a critical organism of study. The workshop participants agreed to establish the *Fundulus* Genomics Consortium (FGC), a collaborative network of investigators interested in enhancing the value of *F. heteroclitus* as a model organism for the study of questions in environmental biology by catalyzing the development of genomic resources. Goals of the FGC are (1) to promote the sharing of physical and virtual resources between laboratories working with *Fundulus* species

through an interactive web site and regular meetings including workshops, (2) to work toward the analysis and annotation of the *F. heteroclitus* genome, including specific agreements to facilitate the production of tools for physical mapping, library production and sequencing, and the analysis of gene expression, and (3) to refocus research questions in *Fundulus* biology within the framework of functional genomics. The compilation of this review on status and opportunities for research with *Fundulus* species provides a starting point for this collaboration. Membership in the FGC is by open declaration of interested parties and the membership list, as well as communication with the FGC is maintained through a mail list serve at fundulus_genomics@whoi.edu. The FGC website is being developed as a central repository for the workshop report, initiatives, links and databases of interest for the *Fundulus* community, and currently is located at <http://fgc.uwaterloo.ca/>.

In their consensus statement, the workshop participants emphasized that *F. heteroclitus* is the teleost model for understanding how individuals and populations interact with their environment. The uniqueness of *Fundulus heteroclitus* as a model species derives from the combination of features that no other model shares, including: ease of care and breeding, moderate size, wide distribution, commercial importance (as bait species), extreme euryhalinity, tolerance of hypoxia, eurythermia, population-specific adaptation to toxicants, well understood developmental biology, physiology and molecular biology and genomics. No other teleost species currently under study has this wide a group of beneficial characteristics. Furthermore, these fish are abundant in coastal environments near human population centers and, consequently, are exposed to many of the same stresses that accompany urban development, including increased risk of

exposure to pathogens and contaminants. Thus, while *F. heteroclitus* serves as a powerful teleost model for environmental effects on human health, we currently lack the full suite of genomic tools that would allow us to develop theory, models and data fully to address pressing issues about coastal environmental degradation and animal health. The community of scientists who employ *F. heteroclitus* as an experimental organism should, indeed must, work together if we are to ensure that genomics-based tools are developed and made available at large, for the benefit of science, the environment, and the organisms, including humans, who rely upon a robust global ecosystem.

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Table 1. EST projects among fishes.

Common Name	Scientific Name	No. of ESTs (NCBI)	No. of ESTs (Other Sources)	Reference
cichlids	<i>Astatotilapia burtoni</i>	3,670	4,000	http://hcegs.unh.edu/cichlid/#cDNA
killifish	<i>Austrofundulus limnaeus</i>	91		
goldfish	<i>Carassius auratus</i>	2,688		
common carp	<i>Cyprinus carpio</i>	19,866		
zebrafish	<i>Danio rerio</i>	1,364,471	833,880	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=zfsh
pufferfish	<i>Takifugu rubripes</i>	26,115	25,002	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=fugu
killifish	<i>Fundulus heteroclitus</i>	55,267	53,977	http://genomics.rsmas.miami.edu/funnybase/super_craw3/ http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=killifish
stickleback	<i>Gasterosteus aculeatus</i>	277,098	47,693	http://cegs.stanford.edu/stickleback_EST_sequences.jsp
long-jaw mudsucker	<i>Gillichthys mirabilis</i>	5,107		
short-jaw mudsucker	<i>Gillichthys seta</i>	66		
blue catfish	<i>Ictalurus furcatus</i>	10,766		
channel catfish	<i>Ictalurus punctatus</i>	46,297	44,037	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=catfish
chum salmon	<i>Oncorhynchus keta</i>	40		
rainbow trout	<i>Oncorhynchus mykiss</i>	269,112	244,984	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=r_trout
sockeye salmon	<i>Oncorhynchus nerka</i>	674		

chinook salmon	<i>Oncorhynchus tshawytscha</i>	13,992		
Gulf toadfish	<i>Opsanus beta</i>	624		
Nile tilapia	<i>Oreochromis niloticus</i>	470		
Japanese medaka	<i>Oryzias latipes</i>	344,336	147,802	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=o_latipes
flounder	<i>Platichthys flesus</i>	8,267		
guppy	<i>Poecilia reticulata</i>	14		
Atlantic salmon	<i>Salmo salar</i>	432,888	236,009	http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=salmon
Spotted green pufferfish	<i>Tetraodon nigroviridis</i>	314,823		
swordtail	<i>Xiphophorus maculatus x Xiphophorus helleri</i>	9		
cichlids (All species)			63,000	http://hcgs.unh.edu/cichlid/#cDNA
TOTAL		2,908,861		

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